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THE EFFECT OF CHOLESTEROL FEEDING ON THE DISTENSIBILITY OF THE ISOLATED THORACIC AORTA OF THE RABBIT[1]

BY JAMES T. NICHOL[2]

Abstract

The descending thoracic aortas of control and cholesterol fed rabbits were dissected out. These aortas were used to prepare tension–length diagrams. Cholesterol feeding increased the distensibility at pressures up to 70 mm. Hg, but at pressures above this value no significant change is produced. Cholesterol feeding increases the resistance of the aorta to collapse, i.e. the longer the cholesterol feeding is continued, the greater the magnitude of the negative pressure required to make the vessel segment collapse.

I. Introduction

A principal feature of the aorta is its great degree of distensibility when compared to that of the other parts of the vascular system. Distension of the aorta during systole reduces the pressure peak reached in the circulation; the reserve of blood running out of the aorta during the diastolic interval keeps the pressure from falling as low as it otherwise would during this phase. The distensibility of the aorta helps to protect the peripheral vascular bed from the shock of wide excursions in pressure.

This distensibility depends upon the elastic properties of the component parts of the aorta, namely endothelium, fibrous tissue, elastic tissue, and smooth muscle. The individual and collective roles of these elements have been described in detail by Burton (2). The high degree of distensibility of the aorta appears to be due to the larger proportion of elastic tissue present, reported to be in the range of 30 to 40% (4).

It has been pointed out (2) that there have been frequent misinterpretations of the meaning of pressure–volume curves owing to a lack of knowledge of the complicating effects of the law of Laplace (tangential tension in wall of a

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[2] *Holder of a Senior Graduate Medical Research Fellowship from the National Research Council of Canada.*

cylinder = pressure in cylinder × radius of cylinder). Burton has derived an expression relating the volume distensibility D to Young's modulus Y

$$D = 200/(YA/r_0 - P)$$

where A is cross-sectional area, P is pressure, and r_0 is the unstretched radius. From this equation it will be seen that even if the wall obeys Hooke's law (Y constant) the distensibility D will increase as P becomes larger. The confusion which results when we attempt to relate the sigmoid shaped pressure–volume curves to the behavior of elastic elements in the vessel wall may be avoided if our data are used to construct a tension–length diagram. To construct such a diagram the radius of the vessel is required in addition to the pressure versus volume values (the "tension" being simply the product of the pressure and the radius). Here the knowledge of "r" makes it possible to determine the length of the fibers, i.e. the circumference of the vessel.

The rabbit has been used as an experimental animal in the study of atherosclerosis ever since the original work was done by Ignatowsky in 1908 (5). Lesions produced in the rabbit by adding cholesterol to its diet resemble atherosclerotic lesions in humans, although some workers emphasize the differences (7). The atherosclerotic process produces changes in the component parts of the aortic wall. These have been described by Cowdry (3). There is initially a deposition of lipoid material between the intima and the internal elastic lamina followed by the appearance of lipoid cells. As the cholesterol feeding is continued, the internal elastic lamina splits up and becomes completely disorganized. It is invaded by smooth muscle cells and collagenous fibers, the effect being that there is loss of elastic fibers and an increase in collagenous fibers. This would seem to indicate that an alteration in the distensibility pattern of the thoracic aorta might reasonably be expected in the cholesterol fed rabbit.

II. Apparatus and Method

A stainless steel syringe was constructed with its piston controlled by a micrometer screw gauge head. A steel spring was used to keep the piston in contact with the micrometer head. The syringe was fastened to a plastic trough so that an isolated rabbit aorta might be attached to its tip by means of a metal adapter. This is shown in Fig. 1. Two metal three-way stopcocks were inserted between the syringe and the plastic trough. These served to connect the syringe to a mercury manometer and a reservoir. The reservoir contained a water solution of 0.1% Evans Blue to fill the apparatus when required. The syringe, piston, and all metal joints were lubricated with Dow Corning Silicone Stopcock Grease to prevent leakage. All bubbles were removed from the apparatus. The assembled apparatus is shown in Fig. 2.

To calibrate the syringe, a fine hypodermic needle was attached to it and the water delivered by it when the micrometer head was screwed between two scale divisions was weighed to the nearest tenth of a milligram. In this way the volume of the water delivered by the syringe could be measured accurately.

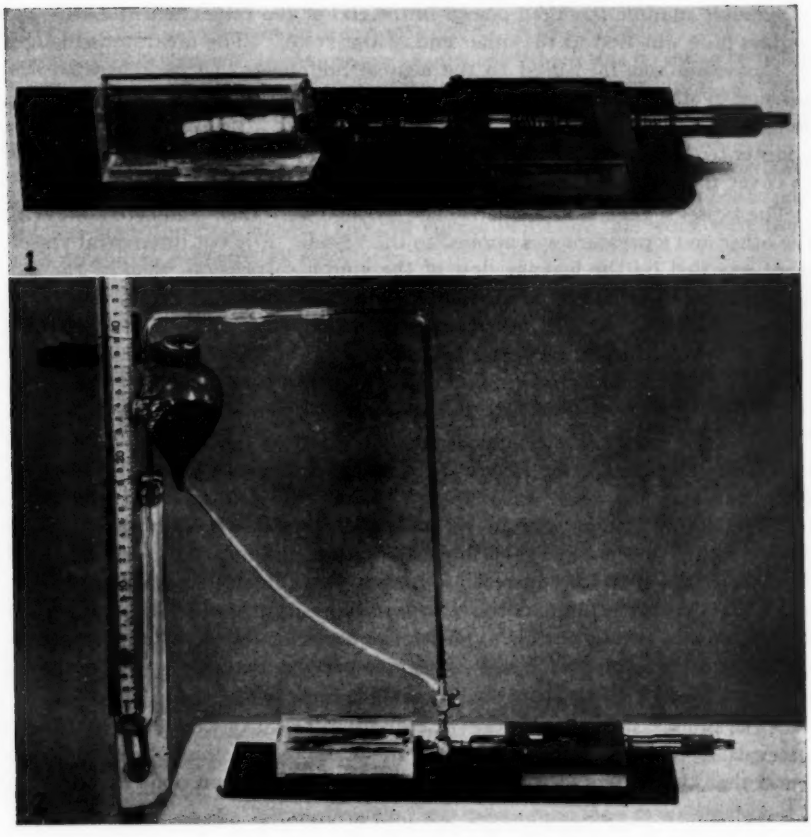

FIG. 1. Steel syringe and plastic trough used to obtain pressure–volume curves of rabbit thoracic aortic segments.

FIG. 2. Apparatus assembly used to obtain pressure–volume curves of rabbit aortas.

The syringe and the manometer alone were then joined and the pressure increased by forcing in the piston. In this way a graph relating micrometer reading and manometer reading was secured. This graph gave a measure of the volume of water required to displace the mercury in the manometer. To get a true measure of the increase in volume of a vessel it was necessary to subtract this 'apparatus baseline' from the apparent increase in volume.

In each experiment the rabbit was killed by urethane and ether and the thoracic aorta was carefully dissected out from the level of the arch to the diaphragm. Care was taken that the intercostal branches of the aorta were not torn but cut cleanly as close as possible to the vertebral column.

A metal adapter was then placed in the end of the vessel near the arch and a glass plug was tied to the other end of the vessel. The unstretched length of the vessel was measured to the nearest millimeter. A concentrated suspension of starch in water was then used to fill the vessel segment, care being taken to expel all air bubbles. The vessel was then attached by means of the adapter to the syringe. The plastic trough was then filled with water sufficient to cover the vessel.

The vessel segment, manometer, and syringe were then connected one with the other and a pressure was applied to the vessel. Any cut intercostal vessels were revealed by the leakage first of the starch suspension, then of the dye solution into the clear water of the trough. The starch served to plug some of the smaller leaks. All other leaks were tied off with black surgical silk (size 4-0). An intact wall was indicated when a pressure of 100 mm. Hg could be maintained for one minute. When this was obtained the vessel was ready for a pressure–volume measurement.

A negative pressure was applied to the vessel to collapse it. When the vessel was collapsed, the 'apparatus baseline' described above was reached, indicating that the vessel was empty. The micrometer head was screwed in by steps and a plot of micrometer values vs. pressure was obtained. Two typical curves done at a 25-min. interval are shown in Fig. 3. It will be noted that there was a sudden increase in volume at a slightly negative pressure which was due to the filling of the collapsed vessel. It reached its undistended volume at zero pressure, which volume can be measured from the curve. At pressures above this value the true pressure–volume curve was obtained (after subtracting the 'apparatus baseline').

In the preparation of these curves individual readings were done at 10-sec. intervals, which were quite sufficient for the pressure to become steady. It was found that the curves so produced were reproducible when done at intervals

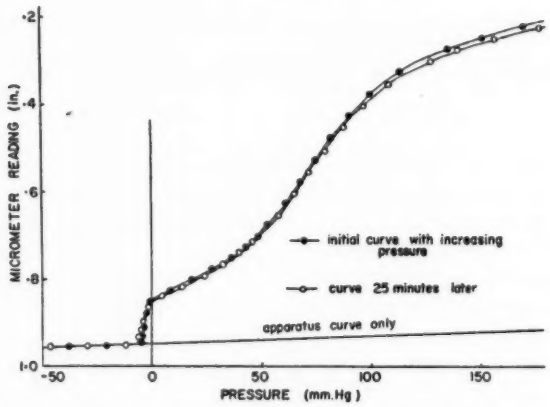

FIG. 3. Typical pressure–volume curves of the thoracic aorta of a normal rabbit.

as close as 10 min. apart. Indeed there was very little change for periods up to 24 hr. if the vessel segment was kept at 5° C. in physiological saline. This would indicate that within the pressure range used there was no overstretching of the vessel to produce any irreversible changes in its distensibility pattern. Curves obtained by reducing the pressure from a higher to a lower value were unsatisfactory as the vessel was unstable, perhaps owing to hysteresis, and the results so obtained were not reproducible. This observation is at variance with those of Alexander, who reports withdrawal curves to be more reproducible than injection curves (1). All curves made for this work were done with ascending pressure values.

The pressure–volume curves were all transformed into tension–length diagrams. This was done by a measurement of the length of the vessel segment in its unstretched condition. The volume $V = r_0^2 \times$ length (where r_0 is the unstretched radius), but if we assume that the length is a constant, then all stretched radii may be determined from a knowledge of changes in V. This assumption may be criticized, but actual measurements showed that the increase in length of a vessel at the greatest distension was less than 10%. However, it will be remembered that r is proportional to V, so that the error in "r" value will be of the order of 5%. The error in the circumference will be correspondingly small. Tensions were thus obtainable from the various values of P and r ($T = Pr$). The lengths (i.e. circumferences) were obtained from the radii at the various pressures. In order to make vessels of different sizes comparable the tensions were plotted against the length as a percentage of its initial unstretched value. The initial length was thus 100% and when the circumference had been doubled the length was considered to be 200%.

One source of error must be acknowledged. Since the ends of the vessel segment were tied to the glass rod and the metal adapter, the vessel segment assumed a sausage shape as the pressure within was increased. Our calculations are based on the assumption that the vessel remained cylindrical, and so r represents the radius of the equivalent cylinder. This error was minimized by making the glass rod and metal adapter as large as possible. Another source of error resulted from the fact that, in the application of the law of Laplace, the internal radius of the vessel was used in obtaining the tension in the vessel wall. The mean radius of the vessel wall was larger than the internal radius because of the thickness of the wall. In actual experiments this error in radius was less than 10% of zero stretch. This factor was of course diminished at higher tensions.

III. Results

A Comparison of the Distensibility of the Thoracic Aorta with That of Rubber Tubing

A tension–length diagram was prepared for the thoracic aorta of the normal rabbit. The diagram has been plotted in Fig. 4. In the same figure is shown

the diagram obtained from a yellow gum rubber tube of the same dimensions. Young's moduli at zero stretch have been derived from these curves. By definition Young's modulus

$$Y = \frac{1}{\text{area}} \times \frac{\text{tension}}{\text{proportional increase in length}}$$

$$= \frac{1}{A} \times \frac{T}{\Delta l / l_0}$$

$$= \frac{100}{A} \times \frac{T}{\% \text{ change in length}}$$

$$= \frac{100}{A} \times \text{slope of graph at origin.}$$

The results so obtained appear in Fig. 4.

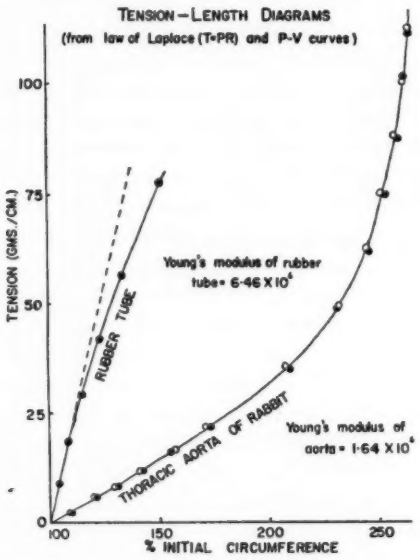

FIG. 4. Tension–length diagrams of rabbit aorta and yellow rubber tubing (Young's moduli have been calculated from the slopes at zero tension).

Effect of Cholesterol Feeding on the Distensibility of the Thoracic Aorta

Ten apparently normal young rabbits were used as controls to prepare a series of pressure–volume diagrams. Ten similar rabbits were fed a 1% cholesterol diet (prepared by spraying solution containing 5% cholesterol on Master's Rabbit Pellets manufactured by Toronto Elevator Company,

mixing, and drying). After they had been fed for periods varying from 78 to 203 days they were killed and the pressure–volume diagrams obtained. Stained sections of the aorta were prepared to ascertain the degree of atherosclerosis. The picture seen was that which had already been described many times by others.

The mean unstretched radius of the aortas of the control animals was $0.146 \pm .005$ cm., while that of the vessels from the cholesterol fed ones was $0.131 \pm .007$ cm. The "P" value for the differences was .073. Hence the cholesterol feeding did not produce a significant change in the unstretched radii of the vessels. An average unstretched radius of 0.138 cm. was thus accepted, and using this as a base value, mean pressure–volume curves were constructed for the two series of rabbits. The results are shown in Fig. 5. The dotted lines indicate the limits of $\pm$ one standard error of the mean.

The results used to construct Fig. 5 were transformed into a tension–length diagram which is shown in Fig. 6.

During the course of the experiments a difference in the behavior pattern of the vessels from the cholesterol fed rabbits as compared to that of the controls was noted. This occurred in the slightly negative pressure range during which collapse of the vessel occurred. In Fig. 7 are shown the typical collapse curves of three controls and the collapse curves of three cholesterol fed rabbits. It will be noted that the longer the cholesterol feeding is continued, the greater is the resistance of the vessel to the collapsing pressure. The mean negative pressures required to produce 80% closure in both control and cholesterol fed rabbits were calculated (80% was selected rather than 100% because of the uncertainty in measuring the latter in a curve which is

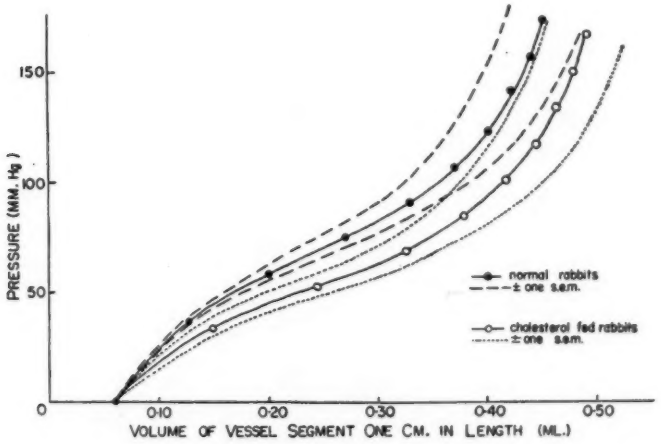

FIG. 5. Mean pressure–volume curves of thoracic aortas from 10 normal and 10 cholesterol fed rabbits.

assymptotic to the zero volume axis). The average pressure for controls was $-3.8 \pm .3$ mm. Hg while in the cholesterol fed ones it was -11.1 ± 1.6 mm. Hg. The standard error of difference between these two means was 1.5. Thus the actual difference was about five times the standard error of difference and P is much less than .01. The difference between the two means was therefore significant.

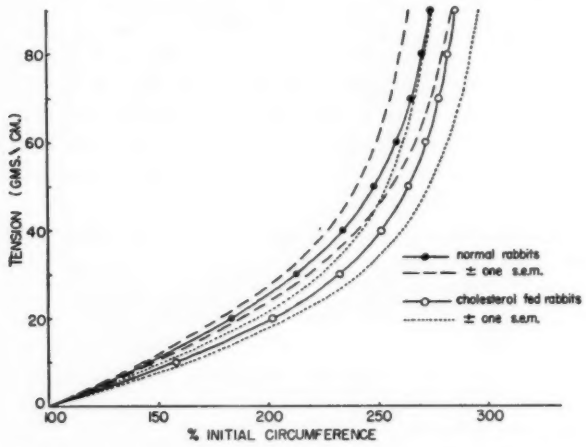

FIG. 6. Mean tension–length diagrams of thoracic aortas from 10 normal and 10 cholesterol fed rabbits.

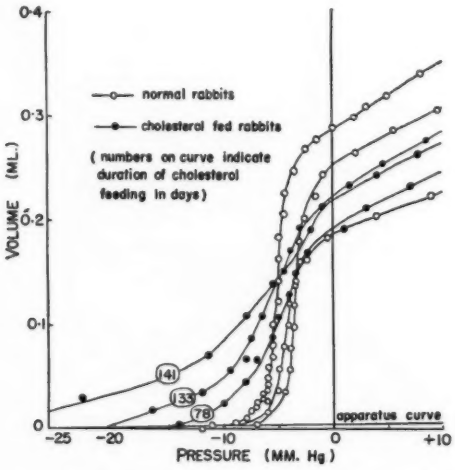

FIG. 7. Collapse curves of thoracic aortas from three normal and three cholesterol fed rabbits.

IV. Discussion

It will be seen in Fig. 4 that the tension–length diagram of the rubber tubing differed from that of the vessel segment. As the rubber was stretched it became more distensible. This, however, was due to the decrease in its cross-sectional area with stretch. Assuming no change in the volume of the rubber, this decrease in area could be calculated. If no decrease had occurred the curve would have been along the dotted straight line. It is reasonable to assume that the elastic tissue component of the vessel should behave in the same way but for the presence of collagenous tissue. As the vessel wall was gradually stretched, more and more collagenous tissue was brought into play, which not only overcame the tendency of the elastic tissue towards greater stretchability but also made the vessel wall less stretchable with stretching. The initial part of the curve probably represents the behavior of the elastic tissue component of the wall while the higher part of the curve represents the situation when the unstretched length ot most or all of the collagenous fibers has been exceeded.

The shape of the curve of the vessel segment may be explained if we assume that the elastic fibers have the function of producing maintenance tension against normal blood pressures and normal pressure fluctuation; the collagenous fibers are stretched only at abnormally high pressures and serve to protect the vessel against irreversible stretching of the elastic tissue and blowout.

The pressure–volume curves for the control and for the cholesterol fed rabbits shown in Fig. 5 are of the sigmoidal type; this shape is in agreement with the findings of other investigators (6, 8, 9). This figure would seem to indicate that atherosclerosis in the rabbit produces a greater degree of distensibility. The same relationship is, of course, indicated in the tension–length diagram in Fig. 6. To determine whether there was a statistically significant difference between the two groups of rabbits, the "*P*" values have been calculated at a series of tension values (see Table I).

These "*P*" values show that there is a significant difference between the two curves up to a tension value of 30 gm./cm. which corresponds to a

TABLE I

MEAN CIRCUMFERENCE OF THE AORTA (AS % OF INITIAL UNSTRETCHED VALUE) ± STANDARD ERROR OF MEAN

Tension (gm./cm.)	10	20	30	40	50	60	70	80	90
Ten control rabbits	145.4 ± .5	183.0 ±4.3	213.0 ±5.4	234.2 ±6.9	249.1 ±8.0	259.2 ±8.9	266.0 ±9.5	271.2 ±9.7	275.1 ±9.7
Ten cholesterol fed rabbits	157.8 ±5.9	202.2 ±7.6	232.9 ±8.5	251.8 ±9.1	264.6 ±9.7	272.8 ±10.1	278.8 ±10.4	283.1 ±10.7	286.5 ±10.7
"*P*" value	.055	.030	.053	.14	.21	.31	.35	.40	.42

pressure of about 70 mm. Hg. Above this value there is no significant difference. In the atherosclerotic process there is a destruction of elastic tissue which explains the greater distensibility in the lower pressure regions. The process includes a proliferation of collagenous tissue which, tissue plays its role at higher pressures. This proliferation apparently compensates for the loss of elastic tissue in the higher pressure regions. The significant change in the curves at lower pressures and the absence of such a change at higher pressures are thus explained by the known changes which occur in the atherosclerotic process.

The greater negative pressures required to collapse the vessels after cholesterol feeding can be explained on the basis of fiber formation in the wall. Such collagenous tissue would resist the deformation necessary for collapse of the vessel.

V. Summary

An apparatus was constructed to measure the pressure–volume relations of the isolated aorta of the rabbit. This information was used to construct tension–length diagrams. At lowest pressures the tension–length diagram is almost a straight line but at higher pressures the slope of the curve becomes steeper. The shape of the tension–length diagram is explained on the basis that the elastic tissue component plays the chief role at low pressures, but that at higher pressures the effect of the less distensible collagenous fibers is superimposed on that of the elastic tissue. The destruction of elastic tissue during the atherosclerotic process induced by a cholesterol diet makes the vessel more distensible at lowest pressures; at pressures above 70 mm. Hg the proliferation of collagenous tissue masks the effect of the loss in elastic material. Also collagenous deposition in atherosclerosis materially increases the resistance of the aorta to the collapse produced by reducing the intraluminal pressure to a negative value.

Acknowledgments

The author wishes to tender his thanks to Dr. A. C. Burton, under whose guidance this work was carried out, and to Mrs. John Elston for technical assistance.

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SOME EFFECTS OF TESTOSTERONE PROPIONATE ON THE IMMATURE PULLET[1]

By D. S. Layne[2] and R. H. Common[3]

Abstract

Sexually immature pullets were treated for 10 days with testosterone propionate (TST) in doses of hormone ranging from 0.25 to 5.0 mgm. per day. TST increased live weight gains, the effect being significant for the highest dosage; TST also increased oviduct weight. Both these results are in agreement with those of previous workers. TST did not affect significantly the net liver weight or the liver weight per kgm. live weight. Liver deoxyribonucleic acid phosphorus (DNAP) per kgm. live weight was not affected by lower dosage levels, but was slightly and significantly increased by higher dosage levels. Liver pentose nucleic acid phosphorus (RNAP) was affected in the same way as liver DNAP; and hence TST had no effect on the ratio RNAP/DNAP in the liver. TST depressed kidney weight, thus confirming previous observations. TST also depressed kidney DNAP and increased kidney RNAP, thereby increasing the ratio RNAP/DNAP in kidney to a marked extent. Spleen weight was depressed by TST, though the effect did not attain statistical significance.

Introduction

The most obvious effect of androgen is stimulation of the development of accessory male reproductive organs, but androgens also influence the skeleton and general conformation of the body. Taber (16) has demonstrated that androgen is produced in the interstitial cells of the medulla of the ovary in the female bird. This androgen is presumably responsible, at least in part, for the hypertrophy of the comb that is seen in the puberal pullet. Androgen may also stimulate growth and increase nitrogen anabolism when administered in suitable amounts to animals, a subject that has been covered by extensive reviews (9, 20). Most of the work relates to mammalian species, but Turner (17) and Kumaran and Turner (11) have shown that suitable doses of androgen may stimulate growth in the chicken. Age and dosage are important factors, for Turner (18) has shown that growth may be depressed if the doses of androgen are insufficient.

Kar (8) and Kumaran and Turner (11) have found that androgen did not affect the size of the chick adrenal, nor did it affect appreciably the weights of the pituitary, thyroid, or parathyroid glands. However, androgen in sufficient dosage will stimulate a slight hypertrophy of the avian oviduct (21, 7, 10). Doses of androgen which, of themselves, are without appreciable effect on the avian oviduct will greatly increase the hypertrophy of the oviduct brought about by a given dose of estrogen (5, 3), an effect which is considered to be a true synergism. A similar synergism in respect of medullary ossification in the bird was noted by Bloom, McLean, and Bloom (1). Others have shown that this synergism also extends to retention of calcium and phosphorus from the food (5, 4).

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Contribution from the Faculty of Agriculture of McGill University, Macdonald College, P.Q. Macdonald College Journal Series No. 369. Supported by a Grant-in-Aid of Research from the National Research Council of Canada.
[2] *National Research Council Bursary for 1953–54.*
[3] *Professor of Agricultural Chemistry.*

The latter experiments, and others reported from this laboratory (11, 13), have been concerned with studies of the effects of concurrent treatments with gonadal hormones designed to simulate the normal puberal changes in gonadal endocrine activity. The experiments have usually involved the treatment of groups of immature pullets with relatively low doses of testosterone propionate (TST) not exceeding one milligram TST per day. Such dosage levels have been found not to exert any evident effects on liver weight or amounts of liver nucleic acids (3, 13) when administered alone. In several unpublished experiments TST has produced a slight but consistent depression of kidney weight; but in general TST alone has not given many positive responses in the pullet as compared with estradiol benzoate (ODB). The infrequency of positive effects of treatment with TST in these experiments might have been a consequence of the low dosage levels used. It was, therefore, decided to study the effects of a fairly wide range of dosage levels of TST (0.25 mgm. to 5.0 mgm. per day) on the liver and kidney of the prepuberal pullet. The experiments included observations on liver and kidney nucleic acid contents.

Experimental

1. Method of Experiment

Twenty-five cross-bred immature pullets (New Hampshire ♂ × Barred Plymouth Rock ♀) were assigned at random to five groups each of five pullets. The birds were 59 days old at the start and 69 days old at the end of the experiment. A commercial poultry starter was fed. Food intake was restricted to 80 gm. per bird per day. One week before the start of the experiment the birds were placed in individual cages situated at random on the laboratory benches.

The treatments are shown in Table I. The birds in group *A* served as controls and received 0.5 ml. of sesame oil daily by intramuscular injection. The birds in groups *B*, *C*, *D*, and *E* received the appropriate dose of TST in 0.5 ml. of sesame oil. The pullets remained in excellent condition, except that those in group *E* (5.0 mgm. TST per day) showed a tendency to molt towards the end of the experimental period.

On the morning following the final injection, the birds were weighed and were then killed by decapitation and bleeding. The ovary, oviduct, spleen, liver, and kidneys of each bird were then removed and weighed. Killing, dissection, and preparations of material for analyses were carried out as quickly as possible, and were completed on each bird before proceeding to the next.

2. General Analytical Methods

(a) Serum Calcium

Serum calcium was determined by diluting 5 ml. of serum with 10 ml. water in a 50 ml. volumetric flask, adding 5 ml. 25% trichloracetic acid slowly and with shaking, making to volume, and mixing. Aliquots of the

filtrate were titrated with ethylene diamine tetracetic acid (EDTA, in the form of "Sequestrene NA2") using murexide indicator as described by Greenblatt and Hartman (6).

(b) Serum Protein

Total serum protein was determined on all samples by the biuret reaction, using the spectrophotometric modification described by Weichselbaum (19).

(c) Preparation of Liver and Kidney Tissue Powders for Nucleic Acid Analyses

Both liver and kidneys (freed from the ureters) were prepared for analysis by pulping in a cylindrical plastic tissue pulper.*

The pulped tissue was disintegrated in 80 ml. ice-cold 10% trichloracetic acid in a Waring blendor. After the addition of some filter-aid ("Celite", Johns-Manville), the precipitate was filtered off on a Büchner funnel and washed with 1% acetic acid. It is important *not* to wash the precipitate with water at this stage. The precipitate should be left in contact with the trichloracetic acid for as short a time as possible, and certainly not overnight.

The precipitate was then re-suspended in about 150 ml. of alcohol–ether (3 : 1 by volume), and subjected to reflux for 10 min. This extraction was repeated. A final extraction was then carried out with a mixture of equal volumes of chloroform and 95% ethanol. The precipitate was finally air-dried, weighed, bottled, and stored in the refrigerator for subsequent analysis for DNAP and RNAP by Schneider's method (15).

(d) Crude Protein

Liver and kidney crude protein (N × 6.25) was determined on separate samples of tissue pulp by micro-Kjeldahl.

(e) Moisture

Approximately one gram of liver or of kidney pulp was dried to constant weight at 105° C. in an air oven.

(f) Determination of Nucleic Acids

The nucleic acids were extracted from the liver tissue powders by the method of Schneider (15). The DNAP in the trichloracetic acid extract was determined by the Dische diphenylamine reaction and the RNAP was determined by the Mejbaum reaction as described by Schneider (15).

Results and Discussion

The results are presented in Tables I and II. From Table I it will be seen that the pullets receiving TST made greater live weight gains than did the control birds, and this effect attained significance in group *E*, which received 5 mgm. TST per bird per day. The effect was not due to stimulation of appetite, because food consumption was held constant for all the birds. Therefore, this effect is an expression of improved utilization of food.

* *Made to the design of Dr. C. Allard of Notre Dame Hospital, Montreal.*

TABLE I

EFFECTS OF TESTOSTERONE PROPIONATE ON THE IMMATURE PULLET—AVERAGE RESULTS

	Groups† and total dosage TST, mgm.					
	A Nil	*B* 10 × 0.25	*C* 10 × 0.75	*D* 10 × 2.0	*E* 10 × 5.0	LSD* (*P* = 0.05)
Live weight, initial, kgm.	0.88	0.85	0.88	0.86	0.86	—
Live weight, final, kgm.	1.24	1.26	1.30	1.28	1.31	—
Increase in live weight, kgm.	0.36	0.41	0.42	0.42	0.45	0.073
Ovary, gm.	0.30	0.32	0.29	0.30	0.31	NS**
Oviduct, gm.	0.15	0.15	0.24	0.28	0.41	0.10
Spleen, gm.	2.77	2.33	2.38	2.24	2.12	0.77
Serum calcium, mgm./100 ml.	10.8	11.0	10.6	11.5	11.2	NS
Serum protein, gm./100 ml.	3.26	3.45	3.16	3.04	3.17	NS
Liver, gm.	23.6	27.0	25.6	22.5	22.5	4.52
Liver, gm. per kgm. live weight	19.0	21.3	19.7	17.7	17.0	2.90
Liver crude protein, %	21.1	21.8	21.0	22.2	21.7	NS
Liver dry matter, %	31.3	32.0	31.8	31.9	31.8	NS
Kidney, gm.	8.14	7.51	7.98	7.05	6.92	1.07
Kidney, gm. per kgm. live weight	6.57	5.94	6.13	5.53	5.26	0.66
Kidney crude protein, %	21.8	22.2	21.7	22.1	22.0	NS
Kidney dry matter, %	21.6	21.1	21.8	21.4	21.5	NS

* *LSD denotes the least significant difference between any two groups by the "F" test.*
** *NS denotes differences non-significant by the "F" test.*
† *Five birds in each group.*

TABLE II

EFFECTS OF TESTOSTERONE PROPIONATE ON NUCLEIC ACID CONTENTS OF LIVER AND KIDNEY OF THE IMMATURE PULLET—AVERAGE RESULTS

	Groups† and total dosage TST, mgm.					
	A Nil	*B* 10 × 0.25	*C* 10 × 0.75	*D* 10 × 2.0	*E* 10 × 5.0	LSD* (*P* = 0.05)
Liver						
DNAP, mgm./100 gm. liver	34.7	31.4	34.4	40.0	42.7	3.6
DNAP, mgm./kgm. live weight	6.58	6.61	6.75	7.02	7.20	0.44
RNAP, mgm./100 gm. liver	77.3	69.5	75.2	86.8	95.3	8.3
RNAP, mgm./kgm. live weight	14.7	14.7	14.8	15.2	16.1	1.3
RNAP : DNAP	2.23	2.22	2.19	2.17	2.23	0.15
Kidney						
DNAP, mgm./100 gm. kidney	44.7	49.3	45.8	49.7	49.5	3.8
DNAP, mgm./kgm. live weight	2.93	2.91	2.80	2.74	2.60	0.27
RNAP, mgm./100 gm. kidney	41.4	46.5	46.9	54.4	59.1	3.9
RNAP, mgm./kgm. live weight	2.71	2.75	2.77	3.00	3.11	0.32
RNAP : DNAP	0.92	0.94	1.03	1.10	1.20	0.09

* *LSD denotes the least significant difference between any two groups by the "F" test.*
† *Five birds in each group.*

No effect on ovary weight was observed.

The two higher levels of TST produced a significant increase of oviduct weight; this result is in agreement with previous observations (21, 7, 10).

The average spleen weight was depressed by increased doses of TST, but this effect did not attain significance by the "*F*" test or by appropriate "*t*" tests. In previous experiments doses of TST up to 1 mgm. per day were found not to affect spleen weight, whereas ODB at the same dosage gave a large and significant depression of weight (12). Although differences here reported for higher doses of TST do not attain significance, the trend of the results suggests that such higher doses may depress spleen weight, but further experimentation will be necessary to establish this point.

Serum calcium was not affected by any level of TST used, a finding in agreement with the results of Riddle and Dotti (14), who found no increase of serum calcium in chicks even after massive dosage with androgen.

Serum protein was not affected by the TST treatments.

Net liver weight was not affected significantly by TST. Liver weight per kgm. live weight in the treated groups was not significantly different from the value in the controls. These results are consistent with comparable results previously secured in this laboratory (3, 12). The differences in percentages of crude protein or dry matter in the liver were small and none attained significance.

In contrast to the results for liver, TST decreased kidney weight and kidney weight per kgm. live weight. These effects were significant ($P = 0.05$) for the second highest dosage rate, and were highly significant ($P = 0.01$) for the highest dosage rate. This observation fully confirms previous observations on this point (12) and emphasizes the difference between the responses of liver and kidney to androgen. The differences in kidney percentages of dry matter and crude protein were small and non-significant.

The results for the nucleic acid contents of liver and kidney are presented in Table II. Liver DNAP per kgm. live weight was not affected by the lower dosages of TST, a result that agrees with previous observations (3, 12). However, the two higher dosage levels significantly increased liver DNAP per kgm. live weight; how far this reflects a hyperplasia induced by higher dosages of TST is uncertain, since the livers were not perfused and the possibility of effects due to differences in amount of residual red cells cannot be excluded. However, the TST treatments in question did not increase liver weight, and the balance of evidence is thought to favor the possibility that the higher doses of TST stimulated some hepatic hyperplasia. The effects of TST on liver RNAP ran closely parallel with the effects on liver DNAP, so that the ratio RNAP : DNAP remained remarkably steady. This result is consistent with the absence of any increase of serum calcium or serum protein, effects which are considered to be related to increased synthesis of serum lipophosphoprotein components by the avian liver (2) under the stimulus of estrogen.

Kidney DNAP per kgm. live weight tended to decrease with increasing dosage rates of TST, and this effect attained significance for the highest level of TST. It is not easy to interpret this result; possibly TST in some way inhibited the normal growth increase in kidney DNAP which probably took place in the control group during the course of the experiment, but this hypothesis is difficult to reconcile with the probable stimulation of hepatic hyperplasia by TST. The conditions of the experiment make it highly unlikely that the result was an artefact, and the point calls for further study. Kidney RNAP per kgm. live weight was significantly increased by the higher levels of TST, so that TST exerted a marked positive effect on the ratio RNAP : DNAP for kidney. This latter result contrasts strongly with the corresponding effects of TST on liver, and suggests that, while TST does not appreciably affect the activity of the liver, it does lead to increased activity of the kidney.

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EFFECTS OF INHIBITORS OF SULPHANILAMIDE ACETYLATION ON SULPHONAMIDE BLOOD CONCENTRATIONS[1]

By Willard Johnson

Abstract

The effect of inhibitors of sulphanilamide acetylation on free and total sulphonamide blood levels has been investigated. *p*-Aminosalicylic acid hydrazide, administered to rabbits in combination with sulphanilamide, produced a twofold increase in the blood level of free sulphanilamide six hours after a single oral dose. Similarly, *p*-aminosalicylate and *p*-(dipropylsulphamyl)-benzoic acid together were responsible for a twofold increase in free-sulphonamide blood level six hours after a single oral dose of a triple sulphonamide mixture. Salicylamide and 5-bromosalicylamide were ineffective. The significance of these results is discussed.

Introduction

Sulphanilamide (*p*-aminobenzenesulphonamide) and its N^1-substituted derivatives are inactivated in the animal body mainly by conjugation with acetate at the N^4-position (7). The rate and extent of acetylation varies with the compound and animal species; the extent of acetylation is apparently the resultant of two processes, namely, acetylation and deacetylation (11). The latter process is extensive in the intact cat, dog, and pigeon, but negligible in man and the rabbit (11).

Inactivation of sulphonamides by acetylation is of some importance in sulphonamide therapy since it represents a decrease in therapeutic effectiveness. In addition to this, there is the danger of kidney damage due to the crystallization of acetylsulphonamide in the kidney tubules (12).

Inhibition of the enzymatic acetylation of sulphanilamide by cyclic carboxamides and carboxhydrazides has been previously reported from this laboratory (8). The present study was planned to test the effectiveness of the more potent of these compounds as inhibitors of sulphonamide acetylation *in vivo*, as evidenced by elevated blood levels of free sulphonamide and, secondarily, by decreased levels of the acetyl derivative.

Methods

Female albino rabbits (2–5 kgm.) were used in most of the experiments. They were maintained on a constant diet of Purina Rabbit Chow (100 gm. per day) containing all vitamins and dietary factors, and water ad libitum. All drugs were given by mouth as a solution or suspension in tap water.

Free and total sulphonamide in blood and urine were determined by the method of Bratton and Marshall (4). A suitable volume of blood, usually 0.2 ml., was transferred by means of a blood pipette to 9 ml. of distilled water. After hemolysis had taken place 3 ml. of 12% trichloracetic acid solution was

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added for deproteinization. Free sulphonamide was determined, immediately after centrifugation, on one 5-ml. portion of the supernatant made up to 10 ml. with water. Another 5-ml. sample of supernatant was hydrolyzed (4), made up to 10 ml. with water, and used for the determination of total sulphonamide. Urine samples, after appropriate dilution, were treated in the same way as blood. Values for total and acetylated sulphonamides are expressed in terms of free sulphonamide. A Coleman spectrophotometer (Model II) was used to determine color intensities.

Results and Discussion

An indication of the extent to which sulphonamides are acetylated in the rabbit is given by the results shown in Table I. It may be seen that more than 80% of the sulphamethazine excreted in the urine in 24 hr. after a single oral dose appeared in the conjugated form. Similar results have been reported by Williams (17) who also observed that when the acetylated drug is fed to rabbits it is excreted unchanged in the urine. Similarly in man, Krebs *et al.* (11) found that, after a single intramuscular injection of sulphamethazine, over 80% of the excreted drug was present in the conjugated form, and after oral administration over 90% was conjugated.

TABLE I

EXCRETION OF SULPHAMETHAZINE IN URINE OF RABBITS

Rabbit No.	Weight (kgm.)	Dosage (mgm./kgm.)	Mgm. excreted in urine in 24 hr.			Conjugated, as per cent of total
			Total	Free	Conjugated	
1	2.10	100	110	28	82	74.5
5	2.13	100	111	12	99	90.0
6	2.00	100	100	14	86	86.0
2	2.38	250	161	25	136	84.5
3	2.35	250	219	27	192	87.7
8	2.00	250	175	30	145	83.0

Following the observation that an occasional rabbit was almost completely lacking in acetylating ability, it became routine practice to test all rabbits as soon as they were received from the supplier in order to eliminate poor acetylators. One such test is shown in Table II. Rabbits numbered 11 and 12 were found to be "non-acetylators", sulphanilamide being present in the blood only in the free form, whilst in rabbits 9, 10, 13, and 14, sulphanilamide was present largely in the conjugated form. Of some interest is the maintenance of a high level of free sulphanilamide in the non-acetylating rabbits; six hours after drug administration, the level had dropped by an average of only 14% as compared with an average of 76.5% for the rabbits which were actively acetylating.

TABLE II

SULPHANILAMIDE BLOOD LEVELS* AFTER A SINGLE ORAL DOSE OF 600 MGM.

Rabbit† No.	Two hours after dosing			Six hours after dosing		
	Total	Free	Acetylated	Total	Free	Acetylated
9	14.9	8.2	6.7	12.9	2.9	10.0
10	19.3	9.6	9.7	12.9	2.2	10.7
11‡	20.5	20.8	0.0	17.5	17.6	0.0
12‡	19.5	19.4	0.1	16.9	16.7	0.2
13	17.1	7.7	9.4	—	—	—
14	14.7	7.3	7.6	12.1	2.6	9.5

* *Mgm. per 100 cc. blood.*
† *All rabbits weighed approximately 2 kgm.*
‡ *Ten days later these were still unable to acetylate.*

The Effect of p-Aminosalicylylhydrazide (PASH) on the Concentration of Sulphanilamide in the Blood

As reported previously (8), *p*-aminosalicylylhydrazide (PASH) at 3×10^{-4} *M* inhibited the *in vitro* acetylation of sulphanilamide by 53% when sulphanilamide was present in the system at 8×10^{-4} *M*. The experimental data presented in Table III suggest that PASH also modifies the acetylation of sulphanilamide *in vivo*. When PASH was administered to rabbits simultaneously with sulphanilamide at half the dosage of the latter, the mean blood level of free sulphanilamide in the PASH-treated rabbits was higher than that of the controls at two, four, and six hour intervals following drug administration (Expt. 1, Table III). The most striking effect was seen at the six-hour interval, when the mean blood level of free sulphanilamide in the PASH-group was more than 300% of the control level.

Experiments 2 and 3 (Table III) taken together represent a crossover type of experiment in which each group of animals served as their own controls. The results of these experiments indicate that PASH produced approximately a twofold increase in the free sulphanilamide blood levels at two and one-half and five hour intervals after dosage.

PASH had a similar effect in rats, though not so pronounced, as would be expected when the poor acetylating ability of the rat as compared with the rabbit is taken into consideration. In one experiment, eight 200-gm. rats were divided into two groups of four. The rats in one group were given, by intubation, 80 mgm. of sulphanilamide; those of the second group received 80 mgm. of sulphanilamide combined with 80 mgm. of PASH. Five hours after drug administration the animals were sacrificed and blood content of sulphanilamide determined. The results, shown in Table IV, indicate that the content of free sulphanilamide in the blood from the PASH-treated rats was 35% higher than that of the control rats. No acetylated sulphanilamide was found in the blood of the PASH-group as compared with 2.2 mgm. % in the control group.

TABLE III

EFFECT OF *p*-AMINOSALICYLYLHYDRAZIDE (PASH) ON SULPHANILAMIDE BLOOD LEVELS IN RABBITS AFTER A SINGLE ORAL DOSE

					Sulphanilamide in blood† (mgm. %)									
					Hours after ingestion of drug									
			Drug dose (mgm.)		2		2½		4		5		6	
Expt.*	Group	No. of animals	Sulphanilamide	PASH	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total
1	—	3	400	—	8.8	15.4	—	—	4.5	14.2	—	—	1.4	7.9
	—	3	400	200	10.9	15.2	—	—	7.0	12.3	—	—	4.4	8.9
2	A	2	600	—	—	—	5.5	12.7	—	—	1.7	7.4	—	—
	B	2	600	500	—	—	10.1	16.4	—	—	3.9	10.8	—	—
3	B	2	600	—	—	—	5.4	15.9	—	—	2.4	9.1	—	—
	A	2	600	500	—	—	10.5	15.8	—	—	5.4	10.4	—	—

* *Experiment 1: Each animal weighed approximately 2 kgm.*
Experiment 2 and 3: Each animal weighed approximately 3 kgm.
† *Each figure represents the mean value for the group.*

TABLE IV

EFFECT OF *p*-AMINOSALICYLYLHYDRAZIDE (PASH) ON SULPHANILAMIDE BLOOD LEVELS IN RATS

No. of rats	Drug dose (mgm./kgm.)*		Sulphanilamide in blood (mgm.%)†	
	Sulphanilamide	PASH	Free	Acetylated
4	400	—	8.5 (7.4–9.5)	2.2 (1.0–3.2)
4	400	400	11.5 (9.5–12.3)	0

* *Drugs were given orally as a suspension in 10% gum acacia.*
† *Mean values. Range is shown in brackets.*

*Effect of Sodium p-Aminosalicylate (PAS) and p-(Di-n-Propylsulphamyl)-benzoic Acid (Probenecid)**

PAS at 2×10^{-3} *M* concentration inhibited sulphanilamide acetylation *in vitro* by 72% (8). When 1 gm. of PAS was administered orally to rabbits along with 500 mgm. of sulphanilamide the blood level of free sulphanilamide, four hours after dosage, was only 141% of the level attained when 500 mgm. of sulphanilamide alone was given. It seems probable that PAS is excreted so rapidly from the rabbit that a concentration of the drug sufficiently high to effectively inhibit sulphanilamide acetylation cannot be maintained in the rabbit unless frequent doses are given. In this connection Venkataraman *et al.* (16) have shown that when PAS is given orally to rabbits, 70–90% of the total amount of PAS recovered in the urine is excreted in the first six hours.

Probenecid, ostensibly by inhibiting the enzymatic combination of PAS with glycine to form para-aminosalicyluric acid (1), is capable of increasing the plasma level of PAS by retarding its renal elimination (3). Probenecid was found to have no inhibitory effect on the acetylation of sulphanilamide by pigeon liver extract. When given to rabbits along with sulphamethazine it was found to increase the blood concentrations of free and acetyl sulphamethazine by 31% and 126% respectively. In view of the evidence that acetylsulphonamides are secreted by the renal tubules (13) the increased blood level of acetylsulphamethazine in the presence of probenecid is likely due to the inhibitory effect of probenecid on renal tubular secretion mechanisms (2). Similar effects of probenecid on acetylsulphonamide levels in humans have been reported by Crosley *et al.* (6), who found that after the intravenous injection of a triple sulphonamide mixture (equal weight of sulphadiazine, sulphamerazine, and sulphamethazine), probenecid produced no change in the blood content of free sulphonamide, but an elevation in the total, which was due to an increase in the acetylated component.

* *Probenecid is the accepted generic name for p-(di-n-propylsulphamyl)-benzoic acid.*

Although probenecid alone did not elevate significantly the free sulphonamide blood level, it was anticipated that the inhibitory effect of PAS on sulphonamide acetylation could be enhanced by utilizing probenecid to delay the renal excretion of PAS. This was found to be the case. Table V shows the results of an experiment in which the control rabbits were given a single oral dose of 750 mgm. of a triple sulphapyrimidine mixture (250 mgm. each of sulphadiazine, sulphamerazine, and sulphamethazine). Another group of three rabbits were given the same dose of triple sulphapyrimidine mixture plus 1 gm. of sodium PAS and 200 mgm. of probenecid. It may be seen from Table V that the blood content of free sulphapyrimidine in the rabbits receiving PAS and probenecid was increased by 121% and 144% at the three and six hour interval, respectively. This increase was accompanied by an increase in the blood level of acetylsulphapyrimidine, which was probably due to the retarding action of probenecid on the renal excretion of acetylsulphonamide.

TABLE V

EFFECT OF SODIUM *p*-AMINOSALICYLATE (PAS) AND *p*-(DIPROPYLSULPHAMYL)-BENZOIC ACID ON THE BLOOD CONTENT OF A SULPHAPYRIMIDINE TRIPLE MIXTURE IN RABBITS

No. of animals	Drug dose	Sulphapyrimidine blood levels (mgm. %)†			
		After three hours		After six hours	
		Free	Total	Free	Total
3	750 mgm. triple sulphapyrimidine mixture*	6.6 (5.5–7.6)	12.5 (11.2–14.2)	2.7 (1.7–3.6)	3.9 (2.1–5.7)
3	750 mgm. triple sulphapyrimidine mixture, 1 gm. sodium PAS, and 200 mgm. probenecid	14.4 (11.7–19.8)	23.5 (21.5–26.0)	6.6 (3.6–10.7)	15.1 (9.1–20.0)

** Triple sulphapyrimidine mixture = 250 mgm. each of sulphadiazine, sulphamerazine, and sulphamethazine. All drugs given as a suspension in tap water.*

† Mean values. Figures between brackets represent range.

We have not investigated the question of product inhibition with regard to the acetylation of sulphonamides. There is a possibility that the elevation of free sulphonamide blood content, observed when probenecid is one of the administered drugs, may be due partially to an inhibition of acetylation resulting from the accumulation of a reaction product, acetylsulphonamide. Product inhibition is a well-known phenomenon as far as *in vitro* enzyme systems are concerned (14), but to our knowledge, its effect *in vivo* has not been reported.

Salicylamide and 5-Bromosalicylamide

Although salicylamide (10^{-3} *M*) and 5-bromosalicylamide (10^{-4} *M*) inhibited competitively the acetylation of sulphanilamide *in vitro* by 56 and 61%, respectively, neither of these compounds influenced sulphonamide blood levels in rabbits. Their lack of effect *in vivo* may possibly be due to their rapid diffusion into the various body tissues (15) and rapid excretion (5).

Isonicotinylhydrazide (Isoniazid)

Isoniazid at 10^{-3} *M* concentration competitively inhibited sulphanilamide acetylation *in vitro* by 56% (8). Moreover, evidence has recently been presented suggesting that isoniazid and sulphanilamide are acetylated by the same enzyme system (9, 8).

Isoniazid was found to be too toxic to be administered to rabbits at the dosage calculated to produce a significant increase in free sulphonamide blood level. However, we have found that sulphanilamide, when given in combination with isoniazid to rabbits, increases the plasma concentration of free isoniazid (10). In these experiments sulphanilamide was given at a considerably higher dosage level than isoniazid.

Therapeutic Implications

It is undoubtedly true that the mean blood level of free sulphonamide and not the total amount of sulphonamide absorbed is the important factor in the effectiveness of oral sulphonamide therapy. The administration of an acetylation inhibitor together with the usual dose of sulphonamide would increase the concentration of free sulphonamide in the blood and presumably would increase the therapeutic effect. Of more clinical importance is the probability that a smaller daily dose of sulphonamide combined with an acetylation inhibitor would be as effective as larger doses of sulphonamide alone.

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ENDOCRINE EFFECTS ON THE HEPARIN-INDUCED LIPEMIA-CLEARING ACTIVITY (LCA) OF RAT PLASMA[1]

By A. Cairns[2] and P. Constantinides

Abstract

A study was made of the relationships of the gonads, the adrenal, and the thyroid to the "lipemia-clearing activity" (LCA) which appears in rat plasma following heparin injection. Strong evidence was obtained that the induction of LCA by heparin is controlled by the estrogenic secretion of the female gonad: Estradiol inhibited LCA; mature females displayed less LCA than mature males, the sex difference being absent in immature animals; ovariectomy and hypophysectomy accelerated LCA; anterior pituitary extracts inhibited it in the female but not in the male; progesterone, testosterone, and orchidectomy had no effect. The adrenal–LCA relationships were complex: Adrenalectomy accelerated LCA but the effects of injected steroids depended on the dosage level and on the duration of the treatment. Short lasting injections of small amounts of glucocorticoids inhibited LCA, the effect disappearing upon prolongation of treatment. By contrast, massive amounts of glucocorticoids accelerated LCA, whether given for a short or a long period of time. Chronic stress and ACTH had no effect, despite the induction of pronounced adrenal enlargement. Injected thyroxine inhibited LCA but surgical thyroidectomy did not affect it.

Introduction

It is well known that heparin clears alimentary lipemia indirectly, i.e. by provoking the *in vivo* formation of a "lipemia-clearing factor" (LCF).

According to recent studies, the LCF is a lipolytic enzyme system activated by heparin. The visible clearing of lipemic turbidity (as induced by heparin *in vivo*, or LCF *in vitro*) is accompanied by a breakdown of large lipoprotein molecules, by a drop in chylomicron count and size, and by fat hydrolysis. In addition, heparin and certain heparinoids diminish *in vivo* the hypercholesterolemia and inhibit the atherosclerosis produced by cholesterol feeding in rabbits (cf. references cited previously (3)).

Since heparin failed to clear the lipemia or reduce the hypercholesterolemia caused by protracted cortisone treatment in the rabbit (2, 3), and since preliminary studies revealed that cortisone or thyroxine pretreatment of heparin-injected rats diminished the lipemia-clearing activity of their plasma (1), it was considered of interest to study systematically the relationships of the gonads, the adrenal, and the thyroid to this lipolytic factor.

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Contribution from the Anatomy Department, University of British Columbia, Vancouver, B.C. The results of this study were presented at the Annual Meeting of the Royal College of Physicians and Surgeons of Canada in Winnipeg, October 22, 1954.

[2] *Third year medical student at the University of British Columbia.*

Materials and Methods

Mature male Wistar rats were used in most experiments of this study, except where gonadal or age effects were the object of interest.

The general procedure followed in each experiment was as follows: Control rats and rats subjected to various endocrine pretreatments were injected with heparin and killed one hour later. The citrated plasma from these heparinized animals was then mixed *in vitro* with citrated lipemic plasma obtained from other rats that had been given an oral oil load three hours previously and the turbidity loss (clearing) of the mixtures was determined colorimetrically at various intervals. It should be emphasized that the lipemia-clearing activities of the plasmata of the control and the experimental groups of every experiment were compared simultaneously, against the same lipemic substrate. Since every heparin-injected group consisted of six to eight animals, and every experiment consisted of two to three groups, the required amounts of lipemic substrate for each performance were obtained by pooling lipemic plasma from several rats.

The specific experimental procedures were as follows: Heparin Connaught (10 mgm./kgm.) was injected subcutaneously into the neck of every control or experimental animal. After 60 min., 4.5 ml. blood was withdrawn from each aorta under ether anesthesia, mixed with 0.5 ml. 0.1 *M* sodium citrate, and centrifuged for 30 min. at 3000 r.p.m. Lipemic plasma was similarly obtained from rats that were given 6 ml. maize oil by stomach tube, three hours prior to sampling. One milliliter of each postheparin plasma was then mixed in a colorimeter tube with 1 ml. lipemic plasma and 3 ml. distilled water. The turbidity of the mixtures was recorded (as logarithmic scale reading) immediately and 20 and 40 min. after mixing, in a Klett-Summerson colorimeter, with a red filter, against a water blank, at room temperature. Since the room temperature and the turbidity of the pooled lipemic substrate varied slightly with each performance, it is obvious that only groups of the same experiment could be compared validly against each other.

All hormones were injected subcutaneously into the caudal dorsal and ventral regions. The following materials and dosages were used: (*a*) *Gonadal.*—Estradiol, 0.2 mgm./rat/day; progesterone, 2 mgm./rat/day; testosterone, 2 mgm./rat/day. All three steroids were given for two weeks. (*b*) *Adrenal.*—Cortisone, 0.25 mgm./rat/day for 7 and 18 days; cortisone, 10 mgm./rat/day for 7 and 18 days; lipoadrenal extract, 1.5 glycogen units/rat/day for 7 and 18 days; desoxycorticosterone, 10 mgm./rat/day for 18 days. (*c*) *Thyroid.*—Thyroxine from tablets, 0.2 mgm./rat/day for 7 days; thyroxine sodium, crystalline, 0.2 mgm./rat/day for 7 days. (*d*) *Pituitary.*—ACTH (Ciba), 6 mgm./rat/day for 10 days; ACTH (Nordic Biochemicals), 4 mgm./rat/day for 10 days; saline extract of acetone-dried beef anterior pituitary powder (Armour), prepared fresh every day, 0.2 gm./rat/day for 7 days. *Chronic cold stress* consisted of exposure to $-2°$ C. for 6 days with ad libitum access to purina and water.

The completeness of hypophysectomy, adrenalectomy, thyroidectomy, ovariectomy, and orchidectomy was verified at autopsy. In addition, in order to assess the specific effectiveness of the various endocrine treatments, some morphological indices of hormone action (or withdrawal) were recorded or measured in most experiments. In the gonadal experiments these criteria were uterine estrus (for estradiol), uterus atrophy (for ovariectomy), progestational endometrial reaction (for progesterone), seminal vesicle hypertrophy (for testosterone), and atrophy (for orchidectomy). End points in the adrenal experiments were the compensatory adrenal atrophy (for injected adrenal steroids), and the adrenal hypertrophy (for ACTH and stress). The action of thyroxine was verified by histological examination of the thyroid gland. The action of anterior pituitary extract was assessed by the adrenal and ovarian enlargement in the female, and the adrenal and seminal vesicle enlargement in the male. Hypophysectomy was judged by the ensuing adrenal atrophy.

It should be noted that, owing to oversight, turbidity readings at the 20 min. interval were omitted in the stress and the male pituitary extract experiment.

Results

A. Effects of Endocrine Pretreatments on the LCA of the Plasma of Heparin-injected Rats

The turbidity loss ("clearing") of the postheparin-lipemic plasma mixtures was expressed in the tables as percentage of the turbidity value of the mixture at mixing or zero time.

1. *Age, Sex, and Gonads (Table I)*
   - (*a*) Senile rats displayed as much LCA as weanlings.
   - (*b*) Mature female rats exhibited much less LCA than mature males.
   - (*c*) There was no sex difference in LCA among immature animals.
   - (*d*) Estradiol markedly inhibited LCA.
   - (*e*) Ovariectomy accelerated it.
   - (*f*) Progesterone, orchidectomy, and testosterone had no significant effects.

2. *Injected Adrenal Steroids (Table II)*
   - (*a*) Small amounts of cortisone or lipoadrenal extract, injected for 7 days, inhibited LCA. Prolongation of the treatment to 18 days led to the disappearance of this inhibitory effect.
   - (*b*) By contrast, massive amounts of cortisone accelerated LCA, whether injected for 7 or 18 days.
   - (*c*) Massive amounts of desoxycorticosterone, injected for 18 days, had no significant effect.

TABLE I

EFFECTS OF AGE, SEX, AND GONADS

Heparin injected group	Mean initial body wt. (gm.)	Mean terminal body wt. (gm.)	Mean % turbidity loss ± ε of postheparin – lipemic plasma mixtures	
			20 min. after mixing	40 min. after mixing
Age differences in female animals				
1 Month old	—	62	23.3 ± 3.0	47.5 ± 3.6
1 Year old	—	280	26.2 ± 2.5	44.0 ± 2.3
Sex difference in immature animals				
1 Month old female	—	62	23.3 ± 3.0	47.5 ± 3.6
1 Month old male	—	64	23.7 ± 2.4	43.3 ± 2.4
Sex difference in mature animals				
Female adults	—	172	25.6 ± 4.5	36.1 ± 4.5
Male adults	—	180	48.2 ± 4.7	55.5 ± 3.4
Male gonad—testosterone				
Controls	155	196	39.7 ± 3.9	48.0 ± 3.4
Orchidectomy	161	192	44.7 ± 3.6	52.7 ± 3.2
Orchidectomy + testosterone	157	187	34.7 ± 3.4	43.0 ± 3.1
Female gonad—estradiol				
Controls	161	179	34.8 ± 2.2	47.2 ± 2.4
Ovariectomy	152	190	41.0 ± 1.6	49.7 ± 1.0
Ovariectomy + estradiol	161	169	15.5 ± 2.7	27.3 ± 3.6
Female gonad—progesterone				
Controls	170	184	51.2 ± 1.1	61.1 ± 0.5
Ovariectomy	160	197	58.1 ± 0.8	61.7 ± 0.6
Ovariectomy ± progesterone	160	204	54.8 ± 1.7	59.5 ± 0.6

TABLE II

EFFECTS OF ADRENAL STEROIDS

Heparin injected group	Mean initial body wt. (gm.)	Mean terminal body wt. (gm.)	Mean adrenal wt. ± ε (mgm./100 gm. terminal BW)	Mean % turbidity loss ± ε of postheparin – lipemic plasma mixtures	
				20 min. after mixing	40 min. after mixing
Cortisone, low dosage (0.25 mgm./rat/day), 7 days					
Controls	145	188	6.3 ± 0.62	26.4 ± 2.3	36.2 ± 2.6
Cortisone	150	189	6.0 ± 0.32	17.3 ± 2.1	28.0 ± 1.3
Cortisone, low dosage (0.25 mgm./rat/day), 18 days					
Controls	144	217	6.0 ± 0.30	39.1 ± 2.4	54.1 ± 1.4
Cortisone	145	213	6.2 ± 0.35	37.9 ± 3.7	52.1 ± 2.0
Lipoadrenal extract (1.5 glycogen units/rat/day), 7 days					
Controls	145	188	6.2 ± 0.62	25.4 ± 2.3	36.2 ± 2.8
Lipoadrenal	151	190	6.3 ± 0.45	16.4 ± 2.5	25.8 ± 2.3
Lipoadrenal extract (1.5 glycogen units/rat/day), 18 days					
Controls	244	313	5.3 ± 0.40	41.5 ± 3.8	58.7 ± 2.8
Lipoadrenal	256	324	5.7 ± 0.50	38.2 ± 2.5	56.0 ± 1.7
Cortisone, high dosage (10 mgm./rat/day), 7 days					
Controls	147	170	7.0 ± 0.65	26.8 ± 2.3	34.6 ± 1.9
Cortisone	139	115	4.3 ± 0.16	35.7 ± 0.9	40.6 ± 1.3
Cortisone, high dosage (10 mgm./rat/day), 18 days					
Controls	244	313	5.3 ± 0.4	41.5 ± 3.8	58.7 ± 2.8
Cortisone	243	212	3.5 ± 0.4	54.0 ± 1.9	63.6 ± 1.0
Desoxycorticosterone, high dosage (10 mgm./rat/day), 18 days					
Controls	260	323	—	40.5 ± 3.9	57.3 ± 2.7
Desoxycorticosterone	242	284	—	37.5 ± 2.8	57.2 ± 1.9

3. *Adrenalectomy, ACTH, and Chronic Stress (Table III)*
   (*a*) Adrenalectomy accelerated LCA.
   (*b*) The two brands of ACTH tested failed to affect LCA.
   (*c*) Chronic stress appeared to have no effect.

4. *Hypophysectomy and Anterior Pituitary Extract (Table III)*
   (*a*) Hypophysectomy (in the female) markedly increased LCA.
   (*b*) Anterior pituitary extract markedly inhibited LCA in the female but it did not seem to have any significant effect in the male.

5. *Thyroid (Table IV)*
   (*a*) Thyroxine (crystalline or from tablets) inhibited LCA.
   (*b*) Thyroidectomy did not affect it.
   (*c*) Combined thyroidectomy–adrenalectomy resulted in no more acceleration of LCA than adrenalectomy alone (compare with Table III).

B. Endocrine Effects on Growth and Specific Target Organs

Only the body and adrenal weight changes were measured and tabulated. Since the marked changes of the sexual organs were visible macroscopically, they were recorded without measurements.

1. *Gonads*

   Growth was not affected by orchidectomy, testosterone, or progesterone but it was accelerated by ovariectomy and retarded by estradiol. Orchidectomy caused marked atrophy whereas testosterone produced marked hypertrophy of the seminal vesicles. Correspondingly, ovariectomy caused pronounced atrophy whereas estradiol produced extreme hypertrophy, hyperemia, and edema of the uteri. Histologically, the endometrial stroma of the progesterone treated group presented characteristic progestational changes (proliferation, enlargement, and vesiculation of the nuclei of the fibroblasts).

2. *Injected Adrenal Steroids*

   Small amounts of cortisone and lipoadrenal extract had no effect on growth or adrenal weight at either treatment interval. Large amounts of cortisone caused pronounced catabolism and adrenal atrophy at both treatment intervals. Desoxycorticosterone did not affect the body weight but it produced extreme adrenal atrophy (not tabulated).

3. *Adrenalectomy, ACTH, and Stress*

   Adrenalectomy arrested growth and stress elicited catabolism. ACTH (both brands) had no effect on body weight but, like stress, it induced pronounced adrenal hypertrophy. It should be noted that in the case of stress the combined weight of both adrenals was recorded whereas in all other experiments only the left adrenal was weighed.

TABLE III

EFFECTS OF ADRENALECTOMY, ACTH, STRESS, HYPOPHYSECTOMY, AND PITUITARY EXTRACT

Heparin injected group	Mean initial body wt. (gm.)	Mean terminal body wt. (gm.)	Mean adrenal wt. ± ϵ (mgm./100 gm.) terminal BW)	Mean % turbidity loss ± ϵ of postheparin – lipemic plasma mixtures	
				20 min. after mixing	40 min. after mixing
Adrenalectomy, 18 days					
Controls	209	257	—	38.8 ± 2.0	48.4 ± 1.5
Adrenalectomy	230	239	—	46.4 ± 1.6	52.5 ± 0.8
ACTH, 10 days					
Controls	158	192	6.3 ± 0.32	29.5 ± 3.5	45.8 ± 3.1
ACTH (Ciba)	156	176	11.8 ± 0.50	33.4 ± 1.5	47.1 ± 2.0
ACTH (N.B.)	161	195	9.4 ± 0.41	33.4 ± 3.1	48.5 ± 2.7
Stress (cold exposure), 6 days					
Controls	201	205	13.4 ± 1.08	—	46.1 ± 1.6
Stress	206	183	20.0 ± 1.80	—	49.9 ± 1.5
Hypophysectomy, in females, 32 days					
Controls	160	250	6.5 ± 0.54	31.5 ± 4.2	47.9 ± 1.4
Hypophysectomy	144	138	3.5 ± 0.41	53.1 ± 1.4	57.8 ± 0.9
Anterior pituitary extract, in females, 7 days					
Controls	283	290	9.9 ± 0.51	39.3 ± 5.0	55.4 ± 2.3
Ant. pit. extract	278	284	17.4 ± 0.62	21.9 ± 4.7	35.1 ± 4.1
Anterior pituitary extract, in males, 7 days					
Controls	240	244	7.2 ± 0.53	—	43.0 ± 1.0
Ant. pit. extract	242	245	12.6 ± 0.59	—	38.9 ± 1.6

TABLE IV

EFFECTS OF THYROXINE, THYROIDECTOMY, AND THYROIDECTOMY–ADRENALECTOMY

Heparin injected group	Mean initial body wt. (gm.)	Mean terminal body wt. (gm.)	Mean % turbidity loss ± ϵ of postheparin – lipemic plasma mixtures	
			20 min. after mixing	40 min. after mixing
Thyroxine, from tablets (0.2 mgm./rat/day), 8 days				
Controls	175	202	37.4 ± 1.4	46.3 ± 0.6
Thyroxine	178	190	27.8 ± 1.3	39.0 ± 1.1
Thyroxine, crystalline (0.2 mgm./rat/day), 8 days				
Controls	159	168	29.2 ± 3.1	45.9 ± 3.0
Thyroxine	164	160	21.4 ± 1.8	36.8 ± 2.6
Thyroidectomy, 18 days				
Controls	211	249	27.1 ± 2.9	35.7 ± 2.5
Thyroidectomy	192	232	26.4 ± 2.9	36.9 ± 2.2
Combined thyroidectomy–adrenalectomy, 18 days				
Controls	—	—	36.9 ± 0.8	41.0 ± 1.2
Thyroidectomy + adrenalectomy	—	—	41.0 ± 1.4	44.9 ± 1.2

4. *Hypophysectomy and Anterior Pituitary Extract*
Hypophysectomy arrested body growth and caused adrenal atrophy, whereas anterior pituitary extract did not affect the body weight and it produced adrenal hypertrophy, in both sexes. In addition, the extract induced hypertrophy of the ovary and uterus in the female and pronounced enlargement of the seminal vesicle in the male.

5. *Thyroid*
Thyroidectomy had no effect on growth but thyroxine from tablets retarded it, and crystalline thyroxine caused catabolism. Histologically, the thyroid glands of the animals that received crystalline thyroxine presented a picture of hypoactivity (flattening of the follicular epithelium and increased amount and density of the colloid).

Discussion

Our data show that the appearance of "lipemia-clearing-activity" (LCA) in the plasma of heparin-injected rats is under multiple endocrine, but predominantly gonadal, control. The observed endocrine effects appeared to be specific, i.e. they could not be correlated with changes of body weight. Of six conditions that arrested growth or induced catabolism, three (massive cortisone, adrenalectomy, hypophysectomy) increased LCA, two (estradiol, thyroxine) inhibited LCA, and one (stress) had no effect on this process. Conversely, of five conditions that inhibited LCA, three (low dosage cortisone, low dosage lipoadrenal extract, anterior pituitary extract in females) did not affect the body weight, and two (estradiol, thyroxine) arrested growth or induced catabolism.

Strong evidence was obtained that the estrogenic secretion of the female gonad inhibits LCA physiologically. The inhibitory effect of estradiol was in line with the sex difference in mature animals, the absence of a sex difference in immature rats, the acceleration of LCA by ovariectomy and hypophysectomy, and its marked inhibition by anterior pituitary extract in the female. Since anterior pituitary extract produced marked adrenal hypertrophy in both sexes but affected LCA only in the female, and since ACTH did not influence LCA, it seems highly probable that the inhibitory effect of the pituitary preparation was due to its gonadotrophic rather than its corticotrophic action. Furthermore, since orchidectomy, testosterone, and anterior pituitary extract did not affect LCF formation in the male, it seems equally probable that the androgenic secretion of the male gonad was not responsible for the observed sex difference.

These results offer a striking parallel to the known gonad – blood lipid relationships in most animal and the human species. It has thus been found that estrogens produce lipemia whereas androgens do not influence blood lipids in birds and mammals, including man (cf. review by Gertler and Oppenheimer (4)). Future research will have to show whether the lipemia-inducing effect of estrogen is due to an inhibition of the recently discovered

"endogenous" lipemia-clearing factor which appears in the plasma after fat meals and has identical characteristics with the "exogenous" (or heparin-triggered) lipemia-clearing factor (5).

The relationship between the adrenal and LCA proved to be more complex and created some questions requiring additional study.

The inhibitory effect of small amounts of glucocorticoids is supported by the recent report of Seifter and Baeder (6) that cortisone produces an inhibitor of LCA, and this effect may explain the previously reported failure of heparin to clear a lipemia induced by comparable amounts of this steroid (2).

The accelerating effect of adrenalectomy, together with the lack of effect of desoxycorticosterone, would suggest that the glucocorticoid secretion of the adrenal antagonizes LCA physiologically. The disappearance of the inhibitory effect of low-dosage glucocorticoids upon prolonged treatment, and the accelerating effect of massive (non-physiological) amounts of cortisone do not necessarily contradict this assumption. There are several precedents in endocrinology of target refractoriness developing after prolonged steroid treatment and of qualitative reversal of action following drastic change in hormone dosage level.

More difficult to explain, at the moment, is the apparent lack of effect on LCA of treatments expected to elicit endogenous glucocorticoid secretion and which caused pronounced adrenal enlargement in our experiments (chronic stress, ACTH). Since Seifter and Baeder (6) found that acute cold exposure, like cortisone, produced an inhibitor of LCA, it may well be that the action of stress on LCA follows the biphasic pattern of Selye's "General Adaptation Syndrome" (7), whereby many changes of the acute phase (Alarm Reaction) are reversed in the chronic phase (Resistance Stage). It is also possible that chronic stress and ACTH provoked homeostatic adjustment mechanisms which could not operate under conditions of exogenous steroid injections, or that they led to only a mild inhibition of LCA which was no longer detectable at 40 min. after mixing, i.e. at a point well past the maximum velocity of the lipemia-clearing reaction. These and other possibilities will be tested experimentally in an attempt to clarify the relationship between endogenous adrenal secretion and LCA, a problem of obvious clinical significance.

As far as thyroxine is concerned, it is doubtful whether its effect was physiological, since thyroidectomy alone failed to accelerate LCA, and combined thyroidectomy–adrenalectomy did not accelerate it more than adrenalectomy alone.

Work on some of the questions that arose out of this study is now in progress.

Acknowledgments

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ACID PHOSPHATASE HYDROLYSIS OF PHOSPHORIC ESTERS[1]

By G. E. Delory, G. S. Wiberg, and Merle Hetherington

Abstract

The rate of hydrolysis and optimum pH of hydrolysis of seminal fluid acid phosphatase have been studied for a number of phosphoric esters. As the acidity of the substrate increases there is a tendency for the rate of hydrolysis to increase and for the optimum pH to move farther away from neutrality. The increased rate of hydrolysis of phenol phosphates or of substituted phenol phosphates can not be accounted for by phenolase activity.

Introduction

In 1943 Delory and King (1) found that the relative rate of hydrolysis of a number of phosphoric esters by the alkaline phosphatase of dog feces and the pH at which activity was optimal were dependent on the acidity of the ester. This paper reports a somewhat similar investigation with the acid phosphatase of seminal fluid.

Methods

The pK values of the esters were determined by the methods previously described (1).

Determination of Optimum pH for Hydrolysis and Rate of Enzymic Activity for Seminal Fluid

The methods were essentially those previously described (1) except that since, in the experiments now being reported, we were interested in acid phosphatase activity a different buffer system was employed. Seminal fluid was diluted so as to contain two units of enzyme per 4 ml. of solution as determined by the method of Watkinson, Delory, King, and Haddow (8). This usually involved a dilution of about 1 in 2000 so that the effect of contaminating substances must have been very small, and since small hydrolysis times were employed the rates of hydrolysis measured were not far removed from initial velocities. The same substrate concentration was used for each ester.

Four milliliters of the diluted seminal fluid were pipetted into each of five 15 ml. flasks which were then placed in a water bath at 37°. Five test tubes each containing about 5 ml. of the appropriate Michaelis buffer mixture (5), range $\pm$ 2.0 of the pH value determined in a rough preliminary experiment (1), were also placed in the water bath.

A 4 ml. pipette was placed in each tube of buffer solution ready for use. The experiment was begun when the solution had attained the temperature of the bath (about five minutes). Four milliliters of the buffers were pipetted into the flasks containing 4 ml. of phosphatase. Two milliliters of the $M/100$

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Contribution from the Department of Biochemistry, University of Manitoba, Winnipeg, Manitoba.

substrate were then added by means of a graduated pipette. An observer stood by with a stop watch to note the times when the substrate was added to each tube.

The contents were then rapidly mixed and after exactly five minutes, 2 ml. of 5% ammonium molybdate in 15% H_2SO_4 were added. This was pipetted into each flask at the same rate as the substrate had been previously added so that the enzyme was in contact with the substrate for exactly five minutes before its destruction by the addition of the acid ammonium molybdate. The flasks were rapidly cooled and 0.5 ml of aminonaptholsulphonic acid solution was added to each tube. After dilution to the 15 ml. mark, the blue color thus obtained was read in the photoelectric colorimeter.

This procedure measured the optimum pH for each phosphoric ester under comparable conditions. The relative rates of hydrolysis of the several esters were redetermined in an experiment in which all the esters were used simultaneously, each at its optimum pH.

Results

Table I shows the values found for pK, optimum pH, and rate of hydrolysis with each of the substrates studied. For convenience of discussion, esters previously studied are presented in group (*a*) while those not previously investigated are given in group (*b*). The results in the first group show (with the exception of the anomalous rate of hydrolysis obtained for tribromophenol phosphate) that with decreasing acidity of the substrate, the rate of hydrolysis decreases and the optimum pH becomes less acid. The effect of concentration of substrate on the rate of hydrolysis was investigated for each ester but in our experiments, the Michaelis–Menten equation was not followed and consequently the constants could not be determined. In this connection it may be noted that Schønheyder (7) showed that the kinetics of semen acid phosphatase were more complicated than would be expected from the theory of Michaelis and Menten. In their experiments with the alkaline phosphatase of dog feces, Delory and King showed that with this enzyme, the rate of hydrolysis decreased with decreasing acidity of the substrate and the optimum activity took place at a less alkaline pH.

The two sets of results show the same relationship between pK and rate of hydrolysis but whereas in the case of the alkaline enzyme, increase in the pK is associated with a decrease in the optimum pH, the reverse is found to occur with the seminal fluid phosphatase. Restating these comments it may be said that in the case of both enzymes, decreasing the acidity of the substrate led to a decrease in the rate of hydrolysis and a movement of the optimum pH towards neutrality.

The results for seminal fluid acid phosphatase, however, only follow this rule in a general way for the substrates in group (*a*) and not at all for those in group (*b*). Since the latter substrates may be regarded as being more physiological in character, the possibility arises that other enzymes are present in seminal fluid which have different rates of hydrolysis towards these sub-

TABLE I

RELATIVE RATES OF HYDROLYSIS OF PHOSPHORIC ESTERS

Substrates	pK_2	Prostate enzyme		Red cell enzyme
		Optimum pH	Relative rate of hydrolysis (phenol = 1.00) at optimum pH	Relative rate of hydrolysis (phenol = 1.00) at pH 5.0
(*a*) Substrates previously investigated				
p-Bromophenol phosphate	5.44	4.7	1.44	1.32
Dibromophenol phosphate	5.60	4.8	1.44	1.18
o-Bromophenol phosphate	5.7	4.8	1.29	1.16
p-Nitrophenol phosphate	5.7	4.9	1.22	1.16
Phenyl phosphate	5.73	5.0	1.00	1.00
o-Cresol phosphate	6.04	5.0	0.94	0.46
Tribromophenol phosphate	6.10	5.2	1.72	0.46
β-Glycerophosphate	6.34	5.6	0.48	0.007
α-Glycerophosphate	6.44	5.8	0.37	0.07
Ethyl phosphate	6.45	6.3	0.04	0.03
(*b*) Substrates not previously investigated				
Glucose-6-phosphate	6.11*	4.9	0.13	0.06
Fructose-6-phosphate	6.11*	5.2	0.30	0.03
Glucose-1-phosphate	6.13*	4.9	0.32	0.012
Fructose-1, 6-diphosphate	6.31	5.7	0.35	0.08

* *Values taken from Oesper (6).*

strates; this however must remain speculative until it is possible to fractionate the enzyme, although Gomori (2) has demonstrated the presence of a hexosediphosphatase in kidney extracts.

Red Cell Phosphatase

Having studied the behavior of prostatic acid phosphatase towards a number of substrates it was desirable to investigate the behavior of red cell phosphatase under similar conditions. Here, however, certain difficulties arise. First of all, the acid phosphatase content of red cells is only one thousandth of that of the seminal fluid while the total solids are about twenty times greater. Secondly, as King and Abul Fad'l (4) have shown, the pH activity curve is more variable and may show two peaks on the acid side. For these reasons a completely comparative study is not possible. However, pending purification of the red cell phosphatases, the rate of hydrolysis of the esters by this preparation was studied in experiments in which, instead of using the optimum pH, all measurements were carried out at the same pH, namely 5.0.

In view of the large amounts of protein present in the red cell phosphatase, the procedure used in the case of seminal fluid was not applicable so that the following modification was used.

Determination of Rate of Hydrolysis for Red Cell Acid Phosphatase

A suitable amount of blood was centrifuged and the plasma pipetted off. The red cells were washed twice with normal saline and then dissolved in water so as to give a 1 to 10 dilution. The solution was centrifuged to remove insoluble material, and the clear supernatant fluid used for the determination. Two milliliters of acetate buffer pH 5.0 were pipetted into each of two centrifuge tubes together with 2 ml. of a $M/100$ solution of the appropriate phosphoric ester.

The tubes were allowed to stand in a water bath at 37° for two minutes to allow the contents to attain the temperature of the bath. After this time, 0.2 ml. of the red cell solution (prepared as above) was added to one of the tubes and hydrolysis allowed to proceed for exactly 30 min. To each tube 0.8 ml. of 25% trichloroacetic acid was added and 0.2 ml. of the red cell solution to the control tube. After they were mixed, the contents of the tubes were centrifuged and 4 ml. of the supernatant fluids was treated with 0.7 ml. of 5% ammonium molybdate solution in 15% sulphuric acid followed by 0.3 ml. of aminonaptholsulphonic acid. After the tubes had stood 15 min. the colors of the solutions were compared colorimetrically with a similarly treated standard solution.

Results

The results are shown in the last column of Table I, from which it can be seen that the order in which the two acid phosphatase preparations hydrolyze the various esters is, in general, the same except for the anomalous behavior of the two glycerophosphates. The fact that prostatic acid phosphatase hydrolyzes β- to a greater extent than α-glycerophosphate (in contradistinction to the red cell enzyme in which the α isomer is more rapidly hydrolyzed) is of course by now well established.

A possible explanation of the greater rates of hydrolysis obtained with the phenol derivatives might be that a phenolase is present. The following section reports an investigation of this possibility.

Methods Used in Studying Phenolase Activity

Phenolase activity was determined by the method of Huggins and Smith (3) which depends on the measurement of the amount of nitrophenol liberated from *p*-nitrophenol sulphate under standard conditions. In view, however, of the small amounts of hydrolysis found in our experiments, and for greater convenience, a hydrolysis time of 18 hr. was substituted for the 10 hr. period used by these workers.

Rat liver homogenate, known to be a source of phenolase, was prepared as follows: The liver was removed from a freshly sacrificed rat, weighed

rapidly, and then homogenized with five times its weight of ice water in a Waring Blendor for three minutes. The homogenate was then centrifuged for 10 min. and the supernatant fluid was collected.

Results

One milliliter of the rat liver homogenate liberated 62.5 μgm. of nitrophenol in 18 hr. Since the homogenate was a one in five dilution of the liver tissue the activity can be expressed as 12.5 μgm. per mgm. of wet tissue. This figure is comparable to the range given by Huggins and Smith for a 10 hr. incubation period, 3.96–7.96.

The results of a series of experiments for seminal fluid are given in the second column of Table II while the third column shows the milligrams of phenol liberated from $M/200$ phenol phosphate by the same preparation in 30 min.

TABLE II

COMPARISON OF AMOUNT OF NITROPHENOL LIBERATED FROM PHENOL SULPHATE WITH THAT OF PHENOL LIBERATED FROM PHENOL PHOSPHATE

Sample No.	Mgm. nitrophenol liberated per 100 ml. seminal fluid*	Mgm. phenol liberated from phenol phosphate per 100 ml. seminal fluid**
1	0.24	92.4
2	0.22	81.9
3	0.15	90.0
4	0.75	62.0

* *Dilution 1 in 25.*
** *Dilution 1 in 2000.*

Discussion

The amount of hydrolysis of *p*-nitrophenol sulphate by the seminal fluid was very small considering that the time of incubation in this case was 18 hr. Experience has shown that under similar conditions 0.05 M phenol phosphate would be fully hydrolyzed. Furthermore, as shown earlier in this paper, nitrophenol phosphate is only hydrolyzed 30% more rapidly than phenol phosphate by the acid phosphatase of seminal fluid.

The above observations would suggest that the pronounced hydrolysis of phenol phosphates and substituted phenol phosphates, in distinction to the other phosphate esters, is not the result of a phenolase being present in seminal fluid. Although seminal fluid has been found to possess some phenolase activity, it is not sufficient to markedly affect the hydrolysis of phenol phosphate.

Acknowledgment

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THE ANTICOAGULANT ACTION IN BLOOD OF SULPHATED DERIVATIVES OF LAMINARIN[1]

By W. W. Hawkins and A. N. O'Neill

Abstract

Laminarin, a polysaccharide from brown seaweeds, has been sulphated to produce compounds which show anticoagulant activity in blood *in vivo*. Derivatives of sulphamic acid were more active than simple sulphate esters, although the amount of sulphate also affected the activity. When tested in rats one sulphated β-aminoethyl ether derivative was 40–50% as potent as heparin, and one ester about 30%. Tests with rats and dogs indicated no harmful effects in the doses used.

Introduction

Laminarin is a polysaccharide found in marine algae, notably kelps and rockweed. It contains no sulphate. It is composed of D-glucopyranose units joined through carbon atoms 1 and 3 in β-glucosidic linkages, and there are between 20 and 40 glucose units per molecule. The molecular weight is four to eight thousand. (See references by O'Neill (6)).

The preparation of sulphated derivatives of laminarin which exhibited anticoagulant activity in blood *in vitro* has been described by O'Neill (6). This paper reports an investigation in animals concerning the activity of four of the derivatives.

Sulphated derivatives of starch, cellulose, and chitin were found by Piper (7) to induce clumping of platelets and a marked lowering of the count as the clotting time of the blood increased after intravenous injection. Conley, Hartmann, and Lalley (1) observed that the effect of heparin *in vitro* was inversely related to the concentration of platelets. Piper (7) also observed, however, that the intravenous injection of heparin into rabbits did not affect the platelets as did the synthetic materials. It was implied that thrombocytopenia denoted toxicity.

Substances such as these may be chemically similar to at least some pyrogens (2, 8), and it might be expected that some possible toxic effects would be similar to those of pyrogens. Young and his co-workers (10, 11) showed that materials which had pyrogenic activity produced in man and the dog a neutrophilic leucocytosis even in doses too small to affect the body temperature.

During tests of the anticoagulant activity of the laminarin derivatives in rats and dogs, observations were made on platelets and white blood cells, and body temperature was followed in the experiments on dogs.

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Materials, Methods, and Procedures

The substances which were tested may be briefly described as follows. *Preparation 1* contained 1.7 sulphate groups per glucose unit, all in half-ester linkages. *Preparation 2* was a sulphated β-aminoethyl ether derivative. It contained 1.7 sulphate groups per glucose unit, of which approximately 1 was in a sulphamic acid group. *Preparation 3* was the same type of compound as *Preparation 2*. It contained 1.6 sulphate groups per glucose unit, of which approximately 0.5 was in a sulphamic acid group. *Preparation 4* was the same type of compound as *Preparation 1*. It contained 1.2 sulphate groups per glucose unit. All preparations were the sodium salts. The laminarin was extracted from *Laminaria digitata*.

Heparin with a potency of 100 I.U. per mgm. was used as a standard. It was obtained from the Connaught Medical Research Laboratories.

Tests on Rats

Adult female rats which weighed about 200 gm. were used in the tests. The animal was given intraperitoneally 3 mgm. of sodium pentobarbital in 0.5 ml. of saline per 100 gm. of body weight. It was then brought under surgical anesthesia with ether. The skin and fascia in the groin were cut through, the muscles separated, and the region of the inguinal ligament was exposed by blunt dissection. The anticoagulant solution was injected into the femoral or the external iliac vein. The wound was then packed with cotton which had been moistened with saline, and closed with a clamp.

The concentration of the anticoagulants was such that the rat received the required dose in 1 ml. of saline per 100 gm. of body weight. The range of dosage of heparin was from 0.1 to 0.45 mgm. per 100 gm., which produced clotting times up to 15 min. after one half hour. The amounts of the synthetic anticoagulants injected were between 0.3 and 4.0 mgm. per 100 gm. of body weight, the dosage being based upon their activity *in vitro* (6). After an interval of 30 min. these amounts gave clotting times similar to that after heparin. Control experiments in which rats were given 1 ml. of saline per 100 gm. of body wt. were also done. Saline was made up in water freshly distilled from glass, and all anticoagulant solutions were used immediately after preparation.

Previous to injection and at intervals of 30 min. thereafter freely flowing blood was obtained from the tail. In most of the experiments on rats the clotting time after the first half hour was the only one measured. The blood was taken directly from the tail into the well of a microscope slide. Its clotting time was measured by the formation of fibrin in the well and in capillary tubes filled from it while all were kept at 37° C. Counts of the white blood cells, with differentials, and of platelets were also made. After the

sample of blood had been obtained the tail was tied with thread. Later the packing was removed from the site of injection, and the wound was stitched up. The animals recovered rapidly.

Tests on Dogs

Two dogs eight months old and from the same litter were used. The tests were carried out over a period of about two months. *Dog A* was a female, and *Dog B* a male. Most of the tests were done between about 10 a.m. and 4 p.m., and the dogs were quiet during the test period.

Standard heparin, *Preparation 1*, and *Preparation 2* were given in doses of 3, 12, and 11 mgm. per kgm. of body weight respectively. *Preparations 3* and *4* were not tested in dogs. The dose per kgm. was contained in 1 ml. of saline. No attempt was made to work out for the dog the dosages which were equivalent to heparin.

The material to be tested was dissolved in saline, and the fresh solution was given intravenously after a blood sample had been taken. Thereafter at intervals blood samples were removed by venepuncture with needles and syringes coated with liquid paraffin. Blood for the various counts was placed in flasks containing potassium and ammonium oxalate. Clotting time was measured by the capillary tube method at 37° C. on blood taken directly from the syringe. Temperatures were taken at the same level in the rectum at intervals throughout each test. Ordinary diurnal variations in all these values were studied both without injection and after the injection of saline.

Total white cell counts were done by the usual method. Smears for differentials were stained with Wright's stain, and 300 to 600 cells from several slides were counted. Platelets were counted by the method of Lempert (4).

Results

Coagulation Times

Under the conditions of the test the normal clotting time of the blood of rats varied from about one to two minutes. The average on 91 animals was 1.3 min. Values within the same range were obtained from one-half to two hours after the intravenous injection of saline.

Preparations 1, *2*, and *3* in quantities of 1.0 to 1.5 mgm. per 100 gm. of body weight gave clotting times of 5–20 min. in one-half hour, 3–15 min. in one hour, 2–7 min. in one and one-half hours, and 1.5–3 min. in two hours. Doses of heparin of this magnitude prolonged the clotting time to the extent that it could not be estimated over a period of two hours. *Preparation 4* at the higher level of 4 mgm. per 100 gm. increased the clotting time by only 1–1.5 min. in one-half hour.

Fig. 1 shows the relationship between the dosage of the anticoagulants and the clotting time of the blood after 30 min. Each point represents the average of four or five estimations for each dosage level. This approached the linear if the dose was plotted against the logarithm of the clotting time. For the purpose of obtaining estimates this relationship was assumed, and the equations to the lines were written. In them the constant which describes the slope of the line for heparin was 2.6; for *Prep. 2*, 1.18; for *Prep. 1*, 0.84; for *Prep. 3*, 0.45; and for *Prep. 4*, 0.09. On this basis, and compared to heparin as 100 I. U. per mgm., the anticoagulant potencies of these preparations were estimated respectively as 45, 32, 17, and 4 I. U. per mgm.

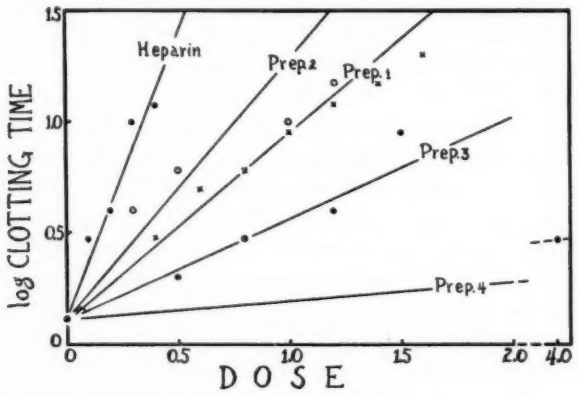

FIG. 1. A comparison of the effect of intravenous injections of heparin and of sulphated derivatives of laminarin on the clotting time of the blood of rats. The clotting time was measured on a sample of blood taken one-half hour after injection of the anticoagulant. Doses are in mgm. per 100 gm. of body weight.

Table I shows examples of the anticoagulant effect of the materials on the blood of dogs. In most cases the clotting time was so prolonged that estimations could be made only after one hour.

Formed Elements of the Blood, and Body Temperature

Table II shows examples from the experiments on rats in which the response to injection of the white blood cells and platelets was studied. The injection of saline caused a decrease in the total white cell count, which was evident in both neutrophiles and lymphocytes. The decrease was greater than that which would result from dilution of the blood with the volume injected. A similar effect was obtained with heparin. Injection of the synthetic anticoagulants was followed by a decrease in neutrophiles and an increase in lymphocytes. There was no consistent trend in platelet counts after any injection, and clumping was not conspicuous at any time.

TABLE I

EFFECT OF ANTICOAGULANTS ON THE CLOTTING TIME OF THE BLOOD OF DOGS

Material injected	Dose (mgm./kgm.)	Clotting time (min.)											
		Initial	½ hr.	¾ hr.	1 hr.	1½ hr.	2 hr.	2½ hr.	3 hr.	4 hr.	4½ hr.	5 hr.	6 hr.
Dog A													
Saline	—	3	3	2	2.5	—	—	—	—	—	—	—	—
Heparin	3	3	—	—	>40	>40	>35	7	—	—	3.5	—	—
Prep. 1	12	—	—	—	>40	33	>15	—	13	6	—	7.5	5.5
Prep. 2	11	3	>35	18	17	—	6.5	4.3	4.5	3	2.7	2.5	2.3
Dog B													
Saline	—	—	—	3	—	—	3	—	—	—	3	—	2.5
Heparin	3	3.7	—	—	>35	>21	24	15	>12	—	—	3	4
Prep. 1	12	4	—	—	>40	18	14	—	11	7.5	—	5	3.8
Prep. 2	11	—	>42	—	20	9.5	5	4	—	—	—	—	—

TABLE II

EFFECT OF ANTICOAGULANTS ON THE WHITE BLOOD CELLS AND PLATELETS OF RATS

Material injected	Dose (mgm./100 gm.)	Time after injection (hr.)	White blood cells (thousands/cu. mm.)			Platelets (thousands/cu. mm.)
			Total	Neut.	Lymph.	
Saline	—	Initial	23.0	2.1	20.0	400
		½	13.5	1.2	11.6	310
		1	10.6	1.5	8.5	340
		1½	15.3	4.6	10.1	350
Heparin	0.3	Initial	9.0	1.0	7.7	533
		½	6.6	0.7	5.6	580
		1	7.3	0.7	6.3	645
Prep. 1	1.7	Initial	16.1	4.5	10.2	480
		½	14.4	1.3	12.7	570
		1	16.4	1.6	14.1	500
Prep. 2	1.5	Initial	17.7	5.0	11.9	570
		½	18.4	3.2	14.5	500
		1	17.8	2.6	14.9	620
		1½	37.4	8.2	28.4	620
Prep. 3	1.2	Initial	12.9	1.9	10.3	430
		½	13.2	1.4	11.5	555
		1½	15.3	3.4	11.4	547
		2	17.7	7.3	10.0	505
Prep. 4	4.0	Initial	9.4	1.5	7.3	440
		½	12.8	1.1	11.2	—
		1	8.7	0.7	7.8	470
		2	9.4	3.3	5.7	—

Table III presents similar observations on *Dog A*, together with observations on rectal temperatures. These data are typical of those which were obtained from both dogs. With the exception of white blood cell counts, fluctuations following the injection of any of the materials were within the range of normal diurnal variations. The rectal temperature was usually highest at the beginning of the experiment. There were considerable fluctuations in temperature, but a higher value than the initial one was rarely shown. There was no trend in the variation of platelet counts, nor was clumping conspicuous at any time. The white blood cell count was usually lowest when the experiment was started. This was also the case with saline injection, and when no injection was given. There was never any indication that fluctuations in the number of cells were due specifically to neutrophiles or lymphocytes.

TABLE III

EFFECT OF ANTICOAGULANTS ON THE PLATELETS AND WHITE BLOOD CELLS AND THE TEMPERATURE OF *Dog A*

Material injected	Dose (mgm./kgm.)	Period of test	No. of blood samples	Range of platelet and white cell counts				Rectal temp.	
				Platelets (thousands/cu. mm.)	Total white cells	Neut. (%)	Lymph. (%)	No. of readings	Range (° F.)
None	—	9.50 a.m.	—	250	10.5	73	16	—	101.2
	—	3.30 p.m.	5	296	17.5	77	22	5	102.8
Saline	—	10.20 a.m.	—	213	7.0	73	10	—	102.5
	—	3.40 p.m.	7	248	12.6	82	19	8	103.8
Heparin	3	9.58 a.m.	—	250	7.8	63	21	—	102.0
	—	3.15 p.m.	6	272	13.7	73	31	6	103.4
Prep. 1	12	10.02 a.m.	—	243	9.4	60	20	—	101.7
	—	4.15 p.m.	8	303	27.4	73	34	8	102.6
Prep. 2	11	9.57 a.m.	—	169	7.6	57	20	—	102.0
	—	4.17 p.m.	9	274	21.3	75	38	8	103.0

Discussion

According to the tests with rats *in vivo*, the anticoagulant *Preparations 2*, *1*, *3*, and *4* are respectively about one half, one third, one fifth, and one twentieth as potent as heparin. These estimations do not differ greatly from those made on the basis of tests with human blood *in vitro* (6). With dogs not sufficient tests were done to make possible a quantitative evaluation.

Of the synthetic anticoagulants which have been reported by others, at least two appear to be more potent than our *Preparation 2*. Chondroitin sulphuric acid (5) and sulphated chitin (9) have been estimated to have respectively 55 and 50 I. U. per mgm. when referred to heparin as 100. Two polysaccharides designated *Mactin-A* and *-B*, which were extracted from clams by workers at the Lederle laboratories (3), were reported to be more active than heparin, and we have confirmed this for *Mactin-A* with rats *in vivo*.

Our preparations showed no pyrogenic action, as indicated by the absence of the sensitive effect on neutrophiles (11) and of rectal hyperthermia. The platelets were not affected in either rats or dogs. With the exception of a possible effect on the total white blood cell count, the compounds appeared to produce no toxic effects in either species.

Further work is being done on the use of some of these preparations as anticoagulants, and on their effects in animals.

Acknowledgments

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THE EFFICACY OF DEXTRANS OF DIFFERENT MOLECULAR WEIGHTS IN SHOCK SECONDARY TO LIMB CLAMPING[1]

By Lorraine C. Smith and R. E. Haist

Abstract

A degree of traumatic shock was produced in rats by the removal of metal clamps which had been applied to both hind limbs for a period of 10 hr. This was lethal within 24 hr. to all animals not receiving fluid therapy. The infusion of plasma expanders delayed or prevented the development of irreversible shock. All fluid therapy relieved the oliguria produced by the shock although this relief generally occurred 24 hr. after the release of the clamps. The plasma expander which best promoted survival was the Connaught dextran 13-1, a solution with an intrinsic viscosity of 0.34 (mean mol. wt. 150,000). Survival with the dextran solutions was increased with increasing molecular weights of the dextrans. This seemed to be related to the retention of the larger molecules for a longer period of time within the body.

Following injury, the development of shock may be prevented in many instances by the early infusion of suitable agents in adequate amounts. The initial aim in therapy is to restore the volume of circulating blood. Most plasma expanders are solutions of relatively inert macromolecular colloids. In general, the increased blood volume is sustained if the molecular weight of the substance is sufficiently large for the substance to remain in the circulation. Renal loss by filtration is the chief pathway for its early removal. At present, dextran appears to be a satisfactory plasma expander and dextran therapy is subject to relatively few complications (4).

The present investigation concerns quantitative measurements of the relative efficacy of several substances in promoting the survival of rats in which shock was produced by limb clamping. The effectiveness of the infusion of several plasma expanders (saline, human serum, Intradex, and three dextrans produced by the Connaught Medical Research Laboratories) was compared in rats subjected to otherwise identical treatment.

Materials and Methods

The experimental animals were female albino rats of the Wistar strain. The average weight of the rats used was 229 gm. (S.D. = 34 gm.). Under light ether anesthesia a fine polyethylene tube was introduced into the right jugular vein for continuous infusion as described by Kinash and Haist (5). The rats were then placed in adhesive tape slings in a constant temperature box maintained at $27.5 \pm 1.0°$ C. where they remained throughout the experiment (5). Ischemia of the hind limbs was produced by the application of broad metal clamps, as described by Haist and Hamilton (2). The clamps were left in position for a period of 10 hr. With this period of ischemia, gangrene of the extremities did not develop. Removal of the clamps after 10 hr. permitted restoration of the circulation to the hind limbs, and shock developed.

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Contribution from Department of Physiology, University of Toronto, Toronto, Ontario.

Infusion was begun as soon as the clamps were removed. A volume of fluid approximately equivalent to the blood volume was infused quickly. A constant rate of 9.0 cc./hr. for two hours was adopted (i.e. approximately 39 cc./kgm. body wt./hr.). The infusion of the test solutions at the same rate into non-shocked rats produced no apparent adverse effects. Syringe type infusion pumps were used. The pumps were connected to the rats by polyethylene tubing with an internal diameter of 0.025 in. One end of the tubing was recurved to form the cannula. Food and water were withheld from all rats in the constant temperature box until 24 hr. after the clamps were removed. Previous experience showed that if water was supplied earlier, the fluid intake introduced too large a variable, which affected survival time.

Accurate survival times were determined by recording the electrical changes associated with the heartbeats of the rats and noting the length of time between removal of the clamps and the stopping of the heartbeats. The arrangement is shown in Fig. 1. Rats were considered to have survived if they lived for 48 hr. At this time the rats were again active and could be kept subsequently for at least a week in ordinary cages.

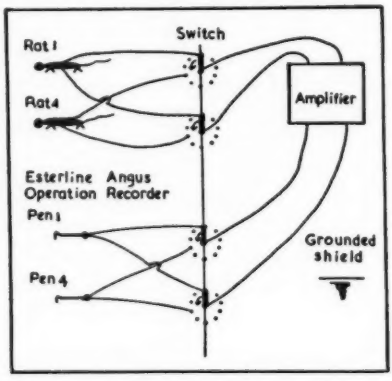

FIG. 1. Simplified diagram of arrangement for recording heart beats, used for the measurement of survival times.

The solutions used for infusion were maintained under sterile conditions. Six different infusion media were tested. These were physiological saline, human serum, Intradex, and three different dextrans from the Connaught Medical Research Laboratories. Table I lists the principal properties of the dextrans used. Three hundred International Units of heparin per 100 cc. were added to all infusion fluids. There was a slight bleeding tendency at the site of the incision in dextran-infused rats.

At specified times (0, 2, 3, 5, 8, 14, 24, 27, 32, 38 and 48 hr.) after the clamps were removed urine samples were collected, the volume measured, and dextran determinations carried out. Initially, a modified anthrone–sulphuric acid method based on that of Durham *et al.* (1) was tried, but this gave unsatisfactory results. Presumably the urinary chromogens caused a

TABLE I

PHYSICAL PROPERTIES OF THE DEXTRAN PREPARATIONS

Properties	Intradex	Connaught dextran		
		Lot 9-1	Lot 13-1	Lot 14-1
Concentration	6%	3.8%	4.4%	5.5%
Intrinsic viscosity	0.315	0.28	0.34	0.30
Molecular weight*	120,000†	100,000	150,000	115,000
Sodium chloride	0.9%	1.05%	0.88%	1.00%
pH	6.5–7.1	6.65	7.00	6.80
Nitrogen, mgm./100 cc.	—	2.0	0.7	0.54

* *Determination based on intrinsic viscosity.*
† *Assuming that Intradex was produced with the same bacterial strain as the Connaught dextrans.*

brown coloration on addition of the anthrone reagent. The turbidimetric method of Metcalf and Rousselot (6) was later tested. This method proved to be reliable and was used in the subsequent investigations.

Results

The survival data on 233 shocked rats are given in Table II. The control rats consisted of two groups. The "untreated" controls were shocked as described. The "sham" controls received a sham operation for the cannulation of the right jugular vein so that they would be comparable to the test animals in every respect, before they also were shocked. The degree of traumatic shock which developed after removal of the metal clamps was lethal to all control animals in less than 24 hr.

TABLE II

SURVIVAL DATA

Treatment	No. of rats	Survivors		Survival time in hours, av. ± S.E.	
		No.	%	Dead rats	All rats*
Controls					
(*a*) Untreated	43	0	0	8.5 ± 0.8	—
(*b*) Sham	46	0	0	5.9 ± 0.6	—
Plasma expanders					
(*a*) Saline	35	6	17.1	12.3 ± 1.8	18.4 ± 2.8
(*b*) Human serum	19	4	21.1	16.0 ± 2.9	22.8 ± 3.8
(*c*) Intradex	21	7	33.3	8.5 ± 2.4	21.6 ± 4.5
(*d*) Dextran 9-1	26	6	23.1	18.2 ± 2.8	25.1 ± 4.0
(*e*) Dextran 13-1	21	10	47.6	20.1 ± 2.7	33.4 ± 3.4
(*f*) Dextran 14-1	22	6	27.3	20.3 ± 2.8	27.8 ± 3.2

* *Survival time of surviving rats taken as 48 hr.*

The survival times for the experimental rats are presented in Fig. 2, which shows that the majority of rats surviving for 30 hr. survived for the duration of the test. The mortality with saline was high. However, it was considerably reduced in rats treated with dextran 13-1 and 14-1. The percentage survival is plotted against time in Fig. 3.

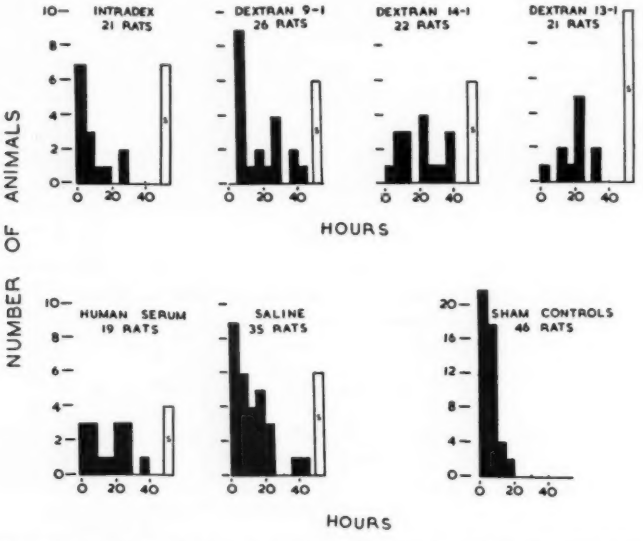

FIG. 2. Frequency distribution of survival times and survival of experimental rats after clamps removed.

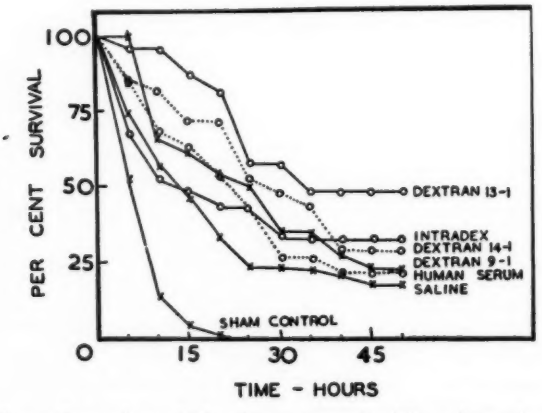

FIG. 3. Comparison of survival, after treatment with various plasma expanders following clamp release.

The various groups are compared for significant differences in Table III (11). Comparison of the number of shocked rats surviving, by means of a chi-square test, shows that all the plasma expanders promoted survival under conditions which were fatal to sham controls. However, only dextran 13-1 led to a significantly greater proportion of survivors than saline. A comparison of the average survival times of the shocked rats by a "t" test indicates that both dextran 13-1 and dextran 14-1 were significantly better than saline.

TABLE III

COMPARISON OF SURVIVAL OF SHOCKED RATS AFTER VARIOUS TREATMENTS

Comparison of treatments		Comparison of No. of shocked rats surviving		Comparison of av. survival times of shocked rats			
				Dead rats		All rats	
		χ^2	p, %	t	p, %	t	p, %
Saline	cf. sham controls	6.20	2	4.04	0.1	5.07	0.1
Human serum		7.00	1	6.98	0.1	6.57	0.1
Intradex		13.74	1	1.54	—	5.07	0.1
Dextran 9-1		8.76	1	6.15	0.1	7.47	0.1
13-1		22.13	1	8.31	0.1	11.35	0.1
14-1		10.58	1	7.56	0.1	9.39	0.1
Human serum	cf. saline	0	—	1.13	—	0.92	—
Intradex		1.13	—	1.25	—	0.65	—
Dextran 9-1		0.06	—	1.84	—	1.55	—
13-1		4.57	5	2.30	2	3.37	0.1
14-1		0.36	—	2.47	2	2.19	5
Dextran 13-1	cf. human serum	—	—	0.99	—	2.08	5
14-1		—	—	—	—	1.03	—
Dextran 13-1	cf. Intradex	—	—	3.20	1	2.09	5
14-1		—	—	3.12	1	1.14	—
Untreated controls cf. sham controls		—	—	2.73	1	—	—

* *Survival time of survivors taken as 48 hr.*

The urine volumes excreted by the various groups of rats over the 48-hr. period are given in Fig. 4. The individual rats in any group receiving the same treatment reacted in a similar manner. Hence the results are presented as averages for the various groups. Eleven "normal" rats excreted urine at a fairly constant rate. Twelve non-shocked rats infused with dextran 13-1 exhibited a slight increase in urinary output over normal rats, and this was maintained over the 48-hr. period. However, non-shocked saline-infused rats showed a diuresis during the first five hours after the infusion was begun. In the shocked rats oliguria was noted during the first 24 hr. after the clamps had been removed. In rats surviving longer than 24 hr., the rate of excretion rapidly increased and approached that of normal animals. The fact that

drinking water was supplied to the rats 24 hr. after the clamps were removed would undoubtedly have some effect on the amount of urine excreted, although there was no evident correlation between the excretion of urine and water intake.

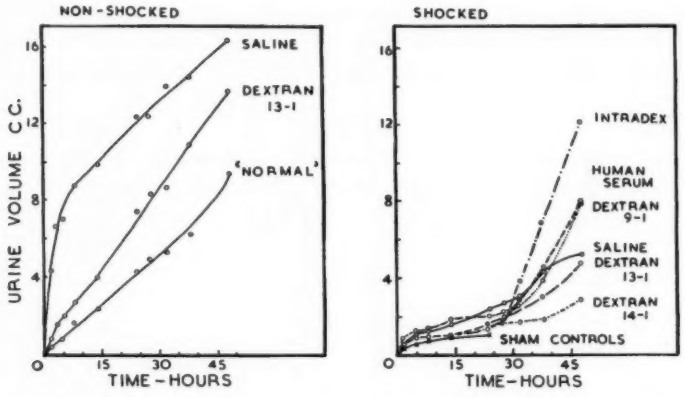

FIG. 4. Total cumulative volume of urine excreted by experimental rats.

According to Raisz (8) and Wasserman and Mayerson (12) the main pathway of dextran excretion is by filtration through the kidney glomeruli, the amount of tubular excretion or absorption being relatively small. The urine of all experimental animals was tested for the presence of dextran (6). Negative results were obtained for all rats except those receiving dextran. The concentration of dextran appearing in the urine is plotted against time in Fig. 5. As the volume of urine excreted by the shocked animal is reduced, the concentration of dextran is increased over that of the non-shocked animal. The concentration of dextran in the urine rises rapidly during the first three to eight hours. It then falls rapidly up to 12 hr. and then more slowly approaches zero at 32 hr. It is interesting to note that the concentration of

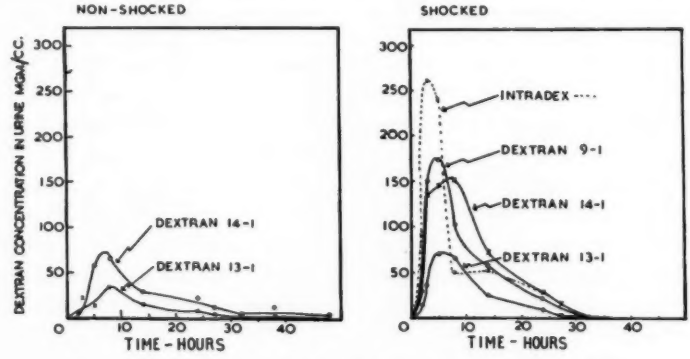

FIG. 5. Concentration of dextran in urine of experimental rats.

dextran reached its highest value in the Intradex-treated rats. This could perhaps be explained by Intradex having a larger molecular weight range, the smaller molecules being rapidly excreted. For the Connaught dextrans, with narrow molecular weight ranges, the concentration in the urine is inversely proportional to the molecular weight of the solution.

Fig. 6 shows that the total dextran excretion by the shocked rats is considerably less than in the non-shocked, dextran-infused rats. Quantitative chemical studies by Mowry and Millican (7) show also that there is a much greater urinary excretion of dextran in normal mice than in mice shocked by burning. Raisz (8) observed the same result in hemorrhagic shock in dogs, although Semple (10) did not. With the Connaught dextrans the total amount of dextran excreted, expressed as a cumulative percentage of the amount infused, is inversely proportional to the molecular weight of the solution. In none of the shocked rats infused with dextran 13-1 did the amount excreted exceed 5% at 48 hr.

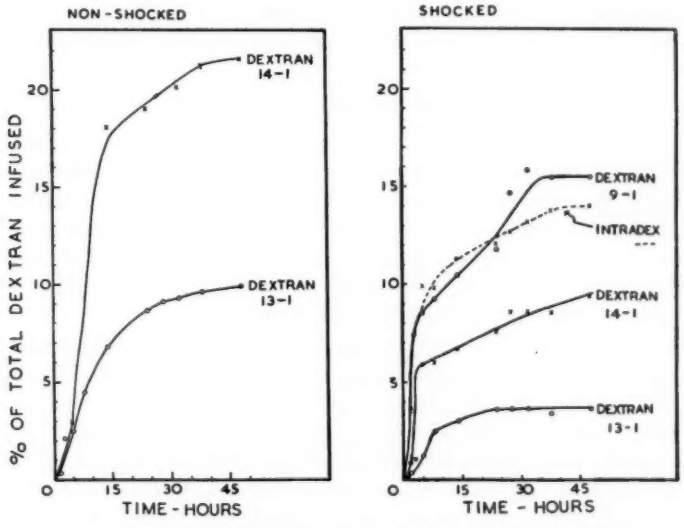

FIG. 6. Cumulative percentage of infused dextran recovered in urine.

The dextran excretion data are compared with the survival data for the various dextran solutions in Table IV. Rats treated with dextran 9-1, the solution with the lowest molecular weight, excreted the most dextran and had the lowest survival of the dextran-treated rats. On the other hand, rats treated with dextran 13-1, the solution with the largest molecular weight, excreted the least dextran and exhibited the best survival response. The large excretion with Intradex may possibly be due to the presence of a larger number of small molecules.

TABLE IV

SURVIVAL AND EXCRETION DATA AFTER INFUSION OF DEXTRAN SOLUTIONS

Treatment	Molecular weight	Mgm. dextran infused	% infused dextran excreted by 48 hr.	% of rats surviving
Non-shocked rats				
Dextran 14-1	115,000	990	21.6	—
Dextran 13-1	150,000	810	10.0	—
Shocked rats				
Dextran 9-1	100,000	720	15.5	23
Dextran 14-1	115,000	990	9.4	27
Intradex	120,000	1080	14.0	33
Dextran 13-1	150,000	810	3.6	48

Discussion and Conclusions

Using a standard clamping technique for the production of shock it was found that the incidence of survival was increased by all the plasma expanders tested and that the development of irreversible shock was delayed or prevented. Solutions of the dextrans with the larger molecular weights (150,000) were more effective in promoting survival than solutions of dextrans with smaller molecular weights. This seems to be related to the better retention of the plasma expander within the blood stream.

It is difficult to compare these results with those of other investigators. This difficulty arises not only because of the different molecular weights of the substances used but also because of the different dosages, rates, and times of infusion and the different methods for measuring dextran excretion. Nevertheless the findings are in essential agreement with those of other authors who have reported an inverse relationship between the amount of dextran appearing in the urine and the molecular size of the preparations infused (Hetzel (3), Ricketts *et al.* (9), Raisz (8)).

Acknowledgments

We are indebted to Dr. Arthur F. Charles of the Connaught Medical Research Laboratories for supplying various dextran preparations and for the determination of the intrinsic viscosity of Intradex. The Intradex preparation was kindly supplied by Glaxo (Canada) Limited. We are grateful also to the Defence Research Board of Canada for supporting this project, which was done under Grant 288.

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IN VITRO STUDIES ON CARBOHYDRATE METABOLISM IN THE VITAMIN-B_6-DEPRIVED RAT[1]

By John R. Beaton

Abstract

Following earlier studies on carbohydrate metabolism in the vitamin-B_6-deprived rat, *in vitro* investigations have been carried out. In all cases, comparisons were made between tissues from vitamin-B_6-deprived and pair-fed control animals so that differences in the amount of food consumed would not affect the interpretation of experimental results. No significant difference was found in glucose utilization by muscle nor in liver cytochrome oxidase activity. Liver aldolase activity was significantly decreased and the activity of plasma alkaline phosphatase was significantly increased in the vitamin-B_6-deprived rats. In vitamin-B_6-deprived female rats, but not male rats, liver catalase activity was significantly increased. These results are discussed in the light of earlier observations indicating disturbances in carbohydrate metabolism in the vitamin-B_6-deprived rat.

Introduction

Two recent studies in this laboratory (2, 3) have demonstrated *in vivo* effects of vitamin B_6 deprivation upon carbohydrate metabolism in the intact rat. The vitamin-B_6-deprived rat has lower fasting blood levels of sugar, lactic acid, and pyruvic acid, lower fasting liver glycogen, and elevated fasting blood and liver levels of inorganic phosphate and glutathione. Blood sugar levels were also studied in these animals following administration of insulin and alloxan (3). In one of these investigations it was found that the activity of liver lactic acid dehydrogenase was significantly lower *in vitro* in deprived rats than in pair-fed controls (2). It is the purpose of this communication to describe the results of investigations on other enzyme systems involved directly or indirectly with carbohydrate metabolism in the vitamin-B_6-deprived rat. The systems studied include glucose utilization by muscle homogenates, liver aldolase, liver catalase, liver cytochrome oxidase, and plasma alkaline phosphatase.

Experimental

In all of the experiments to be reported, Wistar strain rats of both sexes were housed in individual, screen-bottomed cages with drinking water freely available. The basal diet employed was the 20% casein, 20% corn oil, vitamin-B_6-free diet previously described (1). Control animals were given 50 μgm. pyridoxine hydrochloride per rat per day in the food and in all cases were pair-fed with their comparable deprived groups. Following the stated period of experimental feeding, the animals were fasted for 18 hr. and killed by stunning and decapitation. The required tissue was quickly removed, washed, blotted dry, and an aliquot accurately weighed. Following homogenization in a Potter homogenizing tube immersed in ice-water, the homo-

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Contribution from the Department of Public Health Nutrition, University of Toronto, Toronto, Ontario.

genates were stored in an ice-water bath during the short period prior to assay of enzyme activity. Glucose utilization by muscle was measured with the system of Krahl and Bornstein (9) using a 20% homogenate of leg muscle in 0.9% saline (pH 7.45) and an incubation period of five minutes. Liver catalase activity was measured in a 0.4% homogenate by the procedure of Feinstein (7), liver aldolase activity in a 1% homogenate by the method of Dounce, Barnett, and Beyer (6), and liver cytochrome oxidase activity in a 0.5% homogenate by the procedure of Smith and Stotz (11). Using 0.5 ml. of heparinized plasma incubated for 15 min., alkaline phosphatase activity was measured by the method of Bodansky (4).

A "*t*" test has been applied to the results to ascertain the statistical significance of differences between means.

Glucose Utilization by Muscle Homogenates

Fifteen rats were divided into two groups of seven and eight rats with an initial average body weight of 74 gm. One group was deprived of vitamin B_6 and the other group was provided with pyridoxine. Following 34 days of experimental feeding, the animals were fasted and killed for the determination of glucose utilization by muscle homogenates prepared from tissue of the hind legs. The average daily food consumption of both groups was 8 gm. per rat and average body weight gains were 66 and 120 gm. for the deprived and control group respectively. The results of this study are set down in Table I, activities being expressed as μgm. glucose utilized per 100 mgm. tissue per hour.

Liver Aldolase Activities in Vitamin-B_6-deprived Rats

Twenty rats were divided into two groups with an initial average body weight of 62 gm. One group was deprived of vitamin B_6 and the other group was provided with pyridoxine in the food. Following 28 days of experimental feeding, the animals were fasted and killed for the determination of liver aldolase activity. The average daily food consumption of both groups was 9.9 gm. per rat and average body weight gains were 48 and 88 gm. for the deprived and control groups respectively. The results of this study are shown in Table I. Aldolase activities are expressed as mgm. triose phosphate formed per 100 mgm. tissue per hour.

Liver Catalase Activities in Vitamin-B_6-deprived Rats

Eighteen rats were divided into two groups with an initial average body weight of 66 gm., one group being deprived of vitamin B_6 and the other group given pyridoxine. During the 30 day experimental feeding period, the average daily food intake of both groups was 10.0 gm. per rat and average body weight gains were 48 and 95 gm. for the deprived and control groups. The animals were fasted and killed for the estimation of liver catalase activity. The results of this study are set down in Table I. Catalase activities are expressed as milliequivalents sodium perborate destroyed per mgm. tissue per hour.

TABLE I

ENZYME ACTIVITIES IN VITAMIN-B_6-DEPRIVED AND PAIR-FED CONTROL RATS

Experiment No.	Days on diet	Enzyme measured	Enzyme activity*, mean ± S.D.		t
			Vitamin-B_6-deprived	Pair-fed control	
I	34	Glucose utilization by muscle	144 ± 80 (7)†	176 ± 61 (8)	0.88
II	28	Liver aldolase	29.0 ± 3.8 (10)	35.4 ± 7.4 (10)	2.44
III	30	Liver catalase			
		Whole group	5.68 ± 0.67 (9)	5.14 ± 0.84 (9)	1.52
		Females only	5.46 ± 0.63 (4)	4.36 ± 0.26 (4)	3.44
IV	16	Liver cytochrome oxidase	20.8 ± 7.8 (10)	18.9 ± 4.4 (10)	0.68
V	14	Plasma alkaline phosphatase	11.5 ± 1.9 (10)	9.4 ± 2.2 (10)	2.30

* *See text for expressions of activities.*

† *Numbers in parentheses indicate number of animals used.*

Liver Cytochrome Oxidase Activities in Vitamin-B_6-deprived Rats

Twenty rats were divided into two groups with an initial average body weight of 100 gm. and were maintained on experimental feeding for 16 days, one group being deprived of vitamin B_6 and the other group being given pyridoxine. During this time, the average daily food consumption of both groups was 10.9 gm. per rat and average body weight gains were 45 and 54 gm. for the deprived and control groups respectively. The animals were fasted and killed for the determination of cytochrome oxidase activity in liver homogenates. The results are shown in Table I and activities are expressed as μl. oxygen consumed per mgm. tissue per hour.

Plasma Alkaline Phosphatase Activities in Vitamin-B_6-deprived Rats

Twenty rats were divided into two groups with an initial average body weight of 100 gm. and were maintained on experimental feeding for 14 days, one group being deprived of vitamin B_6 and the other group being given pyridoxine. The average daily food consumption of both groups was 12 gm. per rat and average body weight gains were 30 and 42 gm. for the deprived and control groups. The animals were fasted and killed for the determination of alkaline phosphatase activity on heparinized plasma. The results are shown in Table I. Phosphatase activities are expressed in Bodansky units (4) defined as "equivalent to mgm. of phosphorus liberated from sodium glycerophosphate substrate as the phosphate ion during the 1st hour, at pH 8.6 and at 37° C."

Discussion

As emphasized in the earlier studies (2, 3) it should be stressed that, in all cases, comparisons have been made between tissues of pair-fed groups. In this way possible effects of differences in the amounts of food consumed have been eliminated. The only variation in experimental treatment of animals within an experiment is the presence or absence of dietary vitamin B_6.

As shown in Table I, no significant difference was found between the mean glucose utilization by muscle homogenates of vitamin-B_6-deprived and control animals. This finding is of special interest since after only one week of vitamin B_6 restriction, alterations in levels of carbohydrate metabolites, particularly a lowered blood sugar level, are readily apparent. It should be noted that there was a wide individual variation in glucose utilization by muscle homogenates in both groups.

The enzyme aldolase reversibly catalyzes the breakdown of fructose-1,6-diphosphate to 1-phosphodihydroxyacetone and 3-phosphoglyceraldehyde (10). Cori (5) has postulated that the breakdown of fructose-1,6-diphosphate may be limiting in the formation of pyruvate. As shown in Table I, the mean liver aldolase activity of vitamin-B_6-deprived rats was lower than that of the pair-fed controls. This difference was found to be significant at the 5% level. If Cori's postulation is correct (5) then this lower aldolase activity could be a factor in the lower fasting blood pyruvic acid level of vitamin-B_6-deprived

rats (2). However, it should be noted that another liver enzyme involved in pyruvic acid metabolism, lactic acid dehydrogenase, has a similarly lower activity in deprived rats (2).

The physiological role of catalase is in the breakdown of hydrogen peroxide. However, in spite of its extensive study in experimental cancer in animals, its importance in general metabolism is not clear. Since hydrogen peroxide is a by-product of energy production, particularly in the catabolism of carbohydrate metabolites, it might be expected to play a role in carbohydrate metabolism. In this respect, Francoeur and Denstedt (8) recently reported that catalase is responsible for the oxidation of ribose-5-phosphate by human, rat, and rabbit stroma-free hemolyzates of erythrocytes in the presence of ferricyanide under anaerobic conditions. Under aerobic conditions, catalase also oxidized ribose-5-phosphate but this was due to its peroxidase action. In the present study (Table I), an elevated mean liver catalase activity was noted in the vitamin-B_6-deprived group but this elevation failed to attain statistical significance. Considering the female animals only, the mean elevation in liver catalase activity of deprived female rats was found to be significant at the 2% level of comparison with pair-fed female control animals. The interpretation of this elevated liver catalase activity is not clear. At least it can be stated that liver catalase activity is not lowered in vitamin B_6 deprivation and would not be expected to interfere with carbohydrate catabolism.

Cytochrome oxidase, through reoxidation of reduced cytochrome, is an important component of many oxidative systems and is associated with dehydrogenation reactions in both anaerobic glycolysis and oxidative breakdown of carbohydrate. The results of the present study (Table I) demonstrate an insignificant mean increase in liver cytochrome oxidase activity in the vitamin-B_6-deprived group. Considerable individual variation in this enzyme's activity was noted in both groups and application of a "*t*" test showed that within each group, the mean enzyme activity of male rats was significantly less than that of the corresponding female rats. It would appear that, under these experimental conditions, disturbances in carbohydrate metabolism in the vitamin-B_6-deprived rat cannot be attributed to, nor cause, an alteration in liver cytochrome oxidase activity.

In an earlier study (3) it was found that blood and liver levels of inorganic phosphate were significantly elevated in the vitamin-B_6-deprived rat after only one week of vitamin restriction and were still elevated after three weeks. One possible explanation could be a change in the activity of plasma alkaline phosphatase activity. In the present study (Table I), after two weeks of vitamin B_6 deprivation, plasma alkaline phosphatase activities were found to be elevated in the deprived animals (significant at the 5% level) which is consonant with the earlier finding of elevated blood and liver inorganic phosphate levels (3).

These investigations have added support to the hypothesis of an abnormal metabolism of carbohydrate in the vitamin-B_6-deprived rat. Although the

mechanism by which this abnormality occurs has not been fully elucidated, several metabolic alterations have been shown to occur both *in vivo* and *in vitro*. Although *in vitro* findings do not of necessity represent the *in vivo* situation, the present observations on certain enzyme activities appear to substantiate conclusions drawn in previous studies on the intact vitamin-B_6-deprived rat. Further studies are now in progress.

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A COMPARISON OF OPTICAL AND MANOMETRIC METHODS FOR THE ASSAY OF HUMAN SERUM CHOLINESTERASE[1]

By W. Kalow and H. A. Lindsay[2]

Abstract

A method for the assay of human serum cholinesterase (pseudocholinesterase) is described in detail. The disappearance of benzoylcholine is measured by ultraviolet spectrophotometry. Minute amounts of serum and of the substrate are sufficient for a rapid and convenient test of good accuracy. The influence of temperature on the reaction rate is given particular attention, since temperature control is not as easily achieved in the spectrophotometer as for instance in a Warburg apparatus. Although the results of the spectrophotometric assay are fundamentally the same as those obtained by using a conventional gasometric method with acetylcholine as a substrate, small but significant differences between the two answers occur. These differences depend on the substrates and not on the methods as such. The ratio of hydrolysis rates of the two substrates, benzoylcholine and acetylcholine, varies slightly from person to person. If benzoylcholine is used in the Warburg apparatus, a concentration above the optimum must be employed in order to develop sufficient gas for measurement.

Numerous methods to determine the activity of serum cholinesterase (pseudocholinesterase) have been described. Most of these methods were based on one of four principles. Biological assays were first used (13) but are now outmoded. Secondly, the change of pH due to the liberation of acetic acid from acetylcholine was utilized in several ways. Stedman *et al.* (18) introduced a titration technique by adapting a method of Willstaetter *et al.* (19), and Ammon (2) introduced Warburg's method. Most modern standard methods are modifications of one of these procedures. A third principle based on a chemical reaction between hydroxylamine and acetylcholine was introduced by Hestrin (6). It has been important as a research tool because it allows the enzymatic reaction to be studied at various pH's. As a fourth principle, specialized substrates were employed, the hydrolysis of which led to reactive products, e.g. thiocholine (12), indoxyl (3), and beta-naphthol (17). These latter methods were particularly useful for histochemical investigations.

A fifth principle is ultraviolet spectrophotometry, which is well known from work on other enzyme systems (8). It was introduced for investigating the hydrolysis by serum cholinesterase of acetylsalicylic acid (7) and of local anesthetics (9). For the assay of esterase, benzoylcholine serves advantageously as a substrate. Benzoylcholine is hydrolyzed at a great velocity; many data on it are available for comparison (e.g. 1, 5, 11, 14, 15, 16). In the following pages the details of the spectrophotometric assay using benzoylcholine are described and results compared with standard manometric methods.

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[2] *Supported by a grant from the Insulin Fund, University of Toronto.*

Experimental

A. Warburg Experiments

For the experiments in the Warburg apparatus 0.025 molar solution of sodium bicarbonate was used as a solvent. The gas phase consisted of 5% CO_2 in nitrogen. The temperature was 37° C. The fluid volume was 5 ml. The substrate solution was tipped in from the side arm after equilibration. The final concentration of acetylcholine was 2.76×10^{-2} molar, of benzoylcholine 6×10^{-3}. The serum dilution was 1 : 50. Results were corrected for spontaneous hydrolysis and expressed in microliters of CO_2 evolved per 30 min. By multiplication with the factor 0.829 the results indicate micromoles of substrate hydrolyzed per hour per milliliter of serum. The factor $0.829 = 2 \times 50 \times 0.2 \times 1/22.4$; the figure 2 accounts for the time element, the figure 50 for serum dilution, the figure 0.2 for the experimental fluid volume, and the last figure is the ratio of micromoles hydrolyzed to microliters of gas liberated.

B. Optical Method

Equipment

The optical investigations were made in a Beckman spectrophotometer Model DU, with ultraviolet attachment. The wavelength setting of the instrument was checked with a mercury arc. The absorption cells had a light path of 10 mm. For the control of temperature in the cell compartment, the instrument was equipped with thermospacers in connection with a pump and a water bath. The temperature was measured at the inflow and at the outflow from the thermospacers.

Materials

The solvent was always *M*/15 phosphate buffer at pH 7.4, made by dissolving 75.84 gm. of anhydrous Na_2HPO_4 and 18.156 gm. of anhydrous KH_2PO_4 per 10 liters of water. Benzolycholine was obtained commercially as crystalline powder. The source of esterase was diluted human serum. Plasma from citrated or heparinized blood was used on a few occasions until it was found that plasma behaved like serum in our tests.

Basis of Our Method

The optical principle of the spectrophotometric method has been described previously (9). Benzoylcholine has a greater absorbance in the ultraviolet than its split products. In the present series the measurements were made at 240 mμ instead of at 235 mμ, because of the optical properties of dilute serum. The absorbance at 240 mμ of serum diluted 1 : 200 with phosphate buffer is 0.50 with a standard deviation of $\pm$ 0.04; the absorbance at 235 mμ is roughly 1.20. Diluted serum could be kept for several hours in the spectrophotometer at 26° C. without change of absorbance at 240 mμ. Any correction for spontaneous hydrolysis of benzoylcholine was found to be unnecessary.

Procedure

The routine determinations of esterase activity in serum were performed as follows: A solution of benzoylcholine 10^{-4} molar in phosphate buffer was freshly prepared by adding 12.2 mgm. of the chloride to 500 cc. of buffer. A series of reagent tubes were filled with 2 ml. of the solution ("substrate tubes"). For the esterase solution, 0.1 ml. of serum was added to 10 ml. of phosphate buffer. Two or more reagent tubes were each filled with 2 ml. of this solution ("esterase tubes").

To one esterase tube was added 2 ml. of phosphate buffer. This was the optical blank. The instrument was set at zero with this serum blank, using wavelength 240 mμ and a slit width of 1.4 mm.

For the measurements of hydrolysis, the contents of an esterase tube and a substrate tube were rapidly mixed at time zero. The mixture was poured once back and forth, was filled into an absorption cell, and the measurement started 40 sec. after the mixing. One reading was taken thereafter every 20 sec. Readings were continued for four minutes.

Temperature Control

Most experiments were carried out at 26° C., that is, slightly above room temperature. Thereby changes of temperature of the solutions during the mixing were kept at a minimum. Before use and as far as possible during the mixing, the reagent tubes were held in the water bath which was feeding the thermospacers. The cell holder with the absorption cells was always kept in the cell compartment in contact with the thermospacers except for a few seconds for filling. If the experiments were performed between 20° and 26° C., results could be converted into those at 26° by using the formula* antilog $0.0283(t_2-t_1) = v_2/v_1$ (cf. Fig. 1). The temperatures in ° C. are t_1 and t_2, and v_1 and v_2 are the corresponding reaction rates. If, for instance, an optical determination was made at 23.2° C. and the absorbance decreased in three minutes by 0.105, the rate of hydrolysis at 26° would be 0.126 since antilog $0.0283(26.0-23.2) = 1.20 = v_2/0.105$. Results could be converted from 26° C. to 37° C. by multiplication with the factor 1.74. For routine work at 37° C. a serum dilution of 1 : 200 acts too fast for convenience; a dilution of 1 : 400 or 1 : 500 is recommended.

Evaluation and Units

The measurements were plotted on graph paper, the best-fitting straight line was drawn, results were read from the line and expressed as decrease of absorbance per three minutes (ΔA_3). The decrease of absorbance during three minutes was called ΔA_8, if obtained under standard conditions (serum dilution 1 : 200, 5×10^{-5} molar benzoylcholine, 26° C., 10 mm. light path, 240 mμ, pH 7.4).

** As will be shown in a later publication, the rate of reaction measured here is optimal. Then, the rate of reaction $v \simeq k(E)$ where k is a rate constant and (E) the total concentration of esterase. The classical equation relating the temperature coefficient Q_{10} to the rate constants, k_1, k_2 at different temperatures t_1, t_2 is log $Q_{10} = [10/(t_2-t_1)]\log(k_2/k_1)$. We set $\log(k_2/k_1 = \log[k_2(E)/k_1(E)] = \log(v_2/v_1)$. Since $Q_{10} = 1.92$, we arrive by rearrangement of the classical equation at the formula stated in the text.*

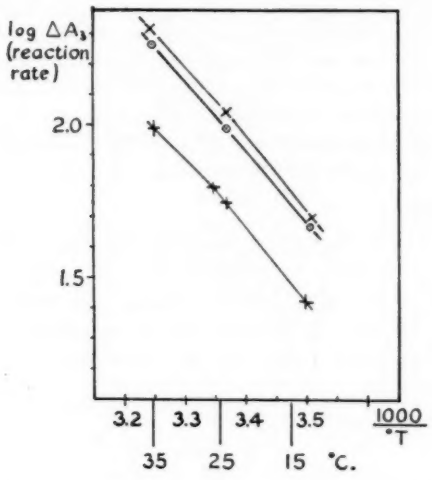

FIG. 1. The influence of temperature on the hydrolysis of benzoylcholine. Independent experiments with three different sera. The log of the temperature coefficient, i.e. log Q_{10}, equals that length of the ordinate which corresponds to an abscissa interval of 10° C. The energy of activation E_a is obtained from the slope of the curves if the reciprocal of the absolute temperature is taken as the scale of the abscissa. E_a = observed slope × 4575, where 4575 = gas constant R × $\log_e 10$ × 1000.

The optical data could be converted to measures of concentration since $0.165\ \Delta A = 2.5 \times 10^{-5}\ M$, that is, a decrease of absorbance of 0.165 corresponds to a hydrolysis of 0.025 micromoles of benzoylcholine per milliliter. Changes of rate are linear with serum dilution, in the range 1 : 1000 to 1 : 100. If procaine is the substrate, there is also linearity in the range 1 : 50 to 1 : 5 (9). It is therefore assumed that the observed rate of hydrolysis can be multiplied by 200 to obtain a measure of the rate of hydrolysis in undiluted native serum. Then, 606 ΔA_s = micromoles of benzoylcholine hydrolyzed by 1 ml. of serum in one hour at 26° C., whereby 606 = (0.025/0.165) × 200 × 20. The factor 20 converts the data from the observed three-minute period to one hour. If one introduces the factor for the temperature conversion (i.e. 606 × 1.74) one obtains 1057 ΔA_s = micromoles of benzoylcholine hydrolyzed by 1 ml. of serum in one hour at 37° C. The value thus derived is larger than would be obtained by measuring the hydrolysis of benzoylcholine in the Warburg apparatus. The reason will be pointed out later.

Frequently used units of serum cholinesterase are defined as micromoles of acetylcholine hydrolyzed by 1 ml. of serum in one hour at 37° C. The optical data can be converted into these units by using the formula $2422\ \Delta A_s - 9.71$, with a standard error of estimate ±15.05. This is an empirical formula calculated by the least squares method from the data which are presented in Fig. 3.

Experimental Error

The same serum was investigated three times on each of three consecutive days by the optical method and with acetylcholine in the Warburg apparatus. The mean value of ΔA_s was 0.0534. The standard deviation was ± 0.00136 or 2.5%. The mean value of the gasometric data was 151.89 μl. CO_2 per 30 min., with a standard deviation ± 5.37 or 3.5%. There was no significant difference between the results on different days.

Studies of Correlation

The esterase activity of 28 persons, including healthy students and patients with liver disease and with hyperthyroidism, was determined by three independent means: Warburg's method was used with acetylcholine and with benzoylcholine, and benzoylcholine was also investigated optically. Figs. 2, 3, and 4 present the data and r the correlation coefficient. The straight lines

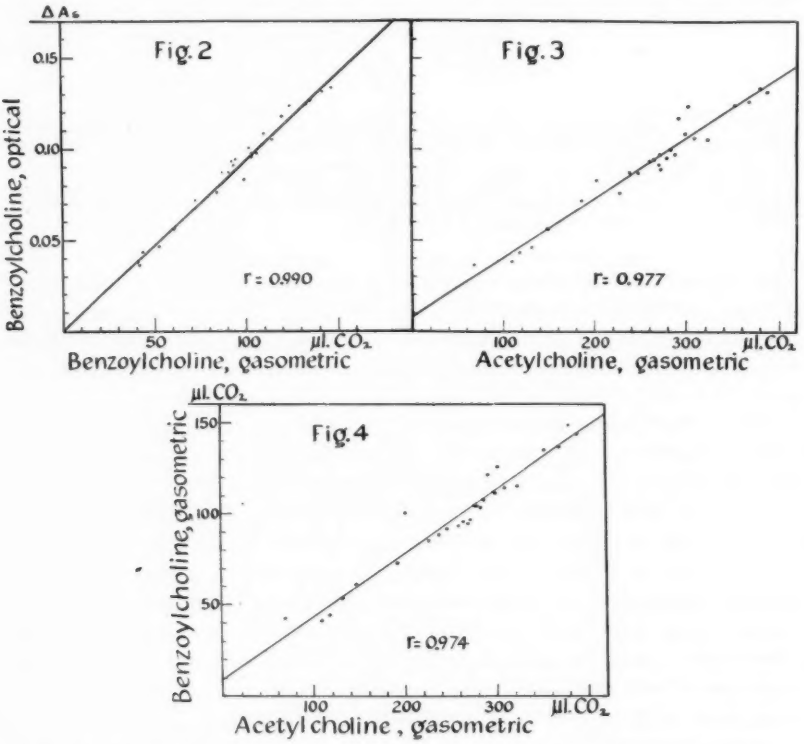

FIG. 2. Scatter diagram. Rates of hydrolysis of benzoylcholine measured optically and in the Warburg apparatus. r = correlation coefficient.

FIG. 3. Scatter diagram. Rates of hydrolysis of benzoylcholine measured optically and of acetylcholine measured in the Warburg apparatus. r = correlation coefficient.

FIG. 4. Scatter diagram. Rates of hydrolysis of acetylcholine and of benzoylcholine, both measured in the Warburg apparatus. r = correlation coefficient.

were calculated by the method of least squares. The data from the gasometric procedures are averages of triplicate determinations, the optical data represent single determinations.

Discussion

If only 0.02 ml. of serum is available for each measurement, the method may be modified by preparing a 5×10^5 molar solution of benzoylcholine, by taking this solution as a blank, by adding the 0.02 ml. of serum directly to 4 ml. of this solution of substrate. If one is willing to have the test take half an hour instead of four minutes, it may be used at 26° C. with 0.002 ml. of serum, or at 37° C. with 0.001 ml. Then water or buffer should be taken as a blank, because of the low absorbance of the highly diluted serum.

The effects of temperature on the rate of hydrolysis of benzoylcholine (Fig. 1) and of procaine (10) are almost identical, while the hydrolysis of acetylcholine is differently affected. In the range from 12° to 26° C. the energy of activation was 11,200 cal. ± 300 degree^{-1} mole^{-1} for benzoylcholine, while it is 7600 cal. for acetylcholine (4).

The three correlation coefficients (Figs. 2 to 4) assure us that all three methods employed are fundamentally measures of the same variable. They all give information on the serum cholinesterase.

The correlation between the optical and gasometric measurements is particularly close if benzoylcholine is the substrate. The rates of hydrolysis of benzoylcholine and acetylcholine, however, are not as closely correlated as is to be expected from the experimental error. If, for instance, the rate of hydrolysis of acetylcholine is estimated from the optically determined rates of hydrolysis of benzoylcholine, the standard error of estimate is 18.2 μl. of CO_2 per 30 min. That is about four times the standard deviation of a triplicate determination of the hydrolysis of acetylcholine, and of the optical procedure.

The same trend can be demonstrated by comparing the correlation coefficients. For instance, the value 0.977 for acetylcholine measured gasometrically and benzoylcholine measured optically differs significantly on the 5% level from the coefficient 0.990 which correlates the two sets of data on benzoylcholine. A comparison of Figs. 3 and 4 confirms the conclusion that the scatter of the data with acetylcholine is not caused by the optical procedure.

Thus there are slight discrepancies between determinations of serum cholinesterase activity depending on the substrate used. Possible causes are a mixture of enzymes, unknown inhibitors, or variable properties of serum cholinesterase itself. It is unlikely that true cholinesterase was present in our sera owing to accidental hemolysis. Had this been the case, the lines of Figs. 3 and 4 should intercept the abscissa rather than the ordinate. So far this intercept is not statistically significant but it also appears in another set of yet unpublished data.

From information supplied in this paper it can be calculated that the hydrolysis of benzoylcholine at 37° C. is roughly 15% faster in the spectrophotometer than in the Warburg apparatus. This depends on the concentrations of benzoylcholine employed, as will be shown in a later publication.

The concentration is 120 times higher in the Warburg apparatus than in the spectrophotometer and is above the concentration giving optimal rates of hydrolysis. This means that the hydrolysis of benzoylcholine is measured in the Warburg apparatus under relatively complicated conditions. This is immaterial for assaying the esterase but it is important for the interpretation of the action of inhibitors of serum cholinesterase.

Summary

The hydrolysis of benzoylcholine in diluted serum or plasma can be measured by ultraviolet spectrophotometry. The method is described in its application for the assay of the activity of serum cholinesterase (pseudocholinesterase) of human serum. If 0.02 ml. of serum is used the measurements take less than four minutes, while the accuracy of the determination is greater than that of a standard test in the Warburg apparatus. The test can be modified to use 0.001 ml. of serum.

The ratio of hydrolysis rates of acetylcholine and benzoylcholine is not constant between sera. Correlation data scatter more than is to be expected from the experimental error.

The rate of hydrolysis of benzoylcholine is slower in the Warburg method than in the spectrophotometric method. The concentration of the substrate is higher in the former and above the optimum.

The energy of activation of the hydrolysis of benzoylcholine is 11,200 $\pm$300 cal. degree^{-1} mole^{-1}.

Acknowledgments

Thanks are due to Prof. J. K. W. Ferguson for his stimulating criticism and to Prof. D. B. W. Reid of the Department of Epidemiology and Biometrics for his statistical advice.

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HYDROLYSIS OF LECITHIN BY PLANT PLASTID ENZYMES[1, 2]

By Morris Kates

Abstract

Enzymatic liberation of choline from egg lecithin by plastid fractions from sugar beet, spinach, and cabbage leaves and from carrot root was a rapid, first order reaction (up to 70% hydrolysis), and was not preceded by a lag phase. None of the choline-containing products of lecithin degradation (lysolecithin, glycerylphosphorylcholine, or phosphorylcholine) lost choline on incubation with spinach chloroplasts. Inorganic phosphate liberation from lecithin by the plastids was preceded by a lag phase and was much slower than choline liberation. Spinach chloroplasts catalyzed the liberation of inorganic phosphate from L-α-phosphatidic acid and from L-α-glycerophosphate. The water-soluble organic phosphate liberated from lecithin by spinach chloroplasts was identified chromatographically as phosphorylcholine. The ether-soluble organic phosphate produced during the hydrolysis of egg lecithin by carrot plastids was isolated and identified as L-α-phosphatidic acid. These observations suggest that the enzymatic hydrolysis of lecithin by plant plastids involves the following reactions: (1) lecithin ⟶ L-α-phosphatidic acid + choline; (2) L-α-phosphatidic acid ⟶ inorganic phosphate + diglyceride and/or (3) L-α-phosphatidic acid ⟶ glycerophosphate + fatty acids and (4) glycerophosphate ⟶ inorganic phosphate + glycerol; and (5) lecithin ⟶ phosphorylcholine + diglyceride. The L-α-structure for egg lecithin was confirmed.

Introduction

Hanahan and Chaikoff (13) found that the release of nitrogenous bases was the only significant enzymatic reaction occurring during the degradation of soybean lecithin by carrot root or cabbage leaf extracts. The ether-soluble product of this degradation, although not isolated, had properties characteristic of a phosphatidic acid, viz., low N/P ratio and acetone-solubility. Acker, Diemair, and Jäger (1) later found that carrot press juice liberated inorganic phosphate as well as choline from yeast lecithin, and also rapidly hydrolyzed phosphorylcholine. They therefore thought that phosphorylcholine might be released first from lecithin and then hydrolyzed to choline and inorganic phosphate. However, when they found that the ratio of inorganic phosphate to choline released was much less than unity, they finally concluded that their enzyme preparation liberated choline first then inorganic phosphate from the resulting phosphatidic acid. Holden (19) has also reported the liberation of phosphate from phospholipids in tobacco chloroplasts.

In a recent study (21), plastid fractions prepared from various plant tissues were found to liberate not only choline but also significant amounts of inorganic and water-soluble organic phosphate from egg lecithin. The ether-soluble products of this degradation have now been examined and have been found to contain organic phosphate. The present paper is concerned with the identification of the water-soluble and ether-soluble organic phosphates, and with the elucidation of the sequence of reactions leading to the observed products of lecithin hydrolysis by plant plastids.

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Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada. Issued as N.R.C. No. 3620.
[2] *Presented in part at the meeting of the American Society of Plant Physiologists, University of Wisconsin, Madison, Wis., September 6–10, 1953.*

Materials and Methods

Plastid Preparations

Spinach, sugar beet, and cabbage chloroplast fractions, and carrot root chromoplast fractions were prepared by the high-speed centrifugation procedure described previously (21). The terms chloroplast or chromoplast fractions used here refer to mixtures of particulate material and not to intact, uncontaminated plastids (see (21)).

Substrates

Phosphatidylcholine (lecithin) was prepared from egg yolk by the method of Hanahan, Turner, and Jayko (15). (Found: 4.15% P, 1.88% N, 16.2% choline; N/P atomic ratio, 1.00; choline N/P atomic ratio, 1.00.)

Lysolecithin was prepared by treatment of egg lecithin (0.41 gm.) with *Naia Naia* venom (1.2 mgm.) in moist ether (40 ml.), following the procedure of Hanahan, Rodbell, and Turner (14). It was precipitated four times from its solution in a minimum amount of chloroform–methanol (2 : 1) by addition of six volumes of moist ether, and was finally washed with anhydrous ether and dried *in vacuo*. (Yield, 0.17 gm., 65%. Found: 6.19% P, 2.71% N, 24.5% choline; N/P, 0.97; choline N/P, 1.01.)

Samples of synthetic dipalmitoyl-L-α-glycerophosphoric acid (phosphatidic acid), the cadmium chloride complex of L-α-glycerylphosphorylcholine (GPC), and the barium salt of phosphorylcholine (PC) were generously provided by Dr. Erich Baer. Barium L-α-glycerophosphate (GP) was prepared by the method of Baer and Fischer (2).

Barium salts of PC and GP were converted to sodium salts by addition of theoretical amounts of sodium sulphate and removal of barium sulphate by centrifugation. The cadmium chloride complex of GPC was converted to the free GPC by treatment with silver carbonate (3).

Analytical Methods

Choline was determined by the reineckate procedure, as described previously (21). Nitrogen was determined by the micro-Kjeldahl method and phosphorus (total P and inorganic P) by King's procedure (22). GP P (total and α-) was analyzed by a modification of Burmaster's periodate method (8), using perchloric acid in place of sulphuric acid, and determining the released inorganic P by King's procedure (22). Total GPC P was also determined by the periodate method, except that the sample was heated for 15 min. at 100° C. in acid solution before addition of periodate.

GP was isolated as the barium salt from phospholipid hydrolyzates by the method of Long and Maguire (24). L-α-GP was estimated enzymatically by the spectrophotometric method of Bublitz and Kennedy (7). An acetone powder of rabbit muscle (18) served as the source of L-α-GP dehydrogenase. An aqueous suspension of the powder (66 mgm./ml.) was dialyzed for 16 hr. and centrifuged, and the clear supernatant was used in the determinations.

Bound fatty acids were determined as follows: the sample (25–50 mgm.) was saponified with 1 *N* sodium hydroxide (2 ml.) on a steam bath for two

hours; the mixture was acidified with 5 *N* hydrochloric acid (0.5 ml.) and extracted several times with low-boiling petroleum ether (35°–55° C.). The ether extract was washed with water, concentrated to dryness in a stream of nitrogen, and the residual fatty acids were dried *in vacuo* and weighed. Neutral equivalents were determined by titrating the solution of the fatty acids in 5 ml. of hot 90% methanol with 0.025 *N* sodium hydroxide (in 90% methanol) to the *o*-cresol red end point.

Phosphate esters (GP, GPC, PC, and lysolecithin) were separated by descending paper chromatography (15 hr., 25° C.) on Whatman No. 1 filter paper (5 × 17 in., acid-washed (28)) with butanol – acetic acid – water (5 : 3 : 1) as solvent. Since the solvent was allowed to run off the paper, position constants for the spots were expressed as

$$R_p = \frac{\text{distance travelled by the compound} \times 100}{\text{distance travelled by orthophosphate}},$$

following the procedure of Mortimer (28).

Activity Measurements

Unless otherwise stated, lecithinase activity (choline and phosphate liberation) was measured, under optimum conditions for choline liberation (21), as follows: the reaction mixture (5.0 ml.), containing acetate buffer (0.1 *M* final concentration), 31–33 μmoles of lecithin, and a suitable amount of plastid fraction, was shaken with diethyl ether (3 ml.) and incubated at 25° C. for a suitable period of time. After 1–2 ml. of 1 *M* trichloracetic acid or 1 *M* perchloric acid was added, the mixture was extracted with ether, and the aqueous phase was analyzed for free choline, total P, and inorganic P.

Glycerophosphatase activity of spinach chloroplasts was measured as follows: to a mixture of 0.5 ml. of 1 *M* acetate buffer (pH 4.7), 1.0 ml. of 0.032 *M* solution of L-α-glycerophosphate, and 1.0 ml. of water was added 2.5 ml. of a spinach chloroplast suspension (containing 70–95 mgm. of plastids). The mixture was shaken and incubated at 25° for 30 min. The reaction was stopped by the addition of 1 ml. of 1 *M* trichloroacetic acid, and the mixture was filtered through Whatman No. 2 filter paper. The filter residue was washed with distilled water and the filtrate was made up to 20 ml. Aliquots of this solution were analyzed for total P, inorganic P, and GP P. The values were corrected for phosphates present in the plastid preparation (blank run without substrate) and activity was expressed as percentage of GP P liberated as inorganic P in 30 min.

Experimental and Results

Kinetic Studies

Time–course curves for the liberation of choline, inorganic P, and water-soluble organic P from egg lecithin by sugar beet, spinach, cabbage, or carrot

plastids are presented in Fig. 1. With all the plastid preparations, liberation of choline was rapid and more than 80% of the choline available from lecithin was set free within one hour. Choline liberation with spinach chloroplasts (Fig. 1*B*) became constant at 83% after 30 min., while that for the other plastids (Fig. 1*A*, 1*C*, 1*D*) approached 100%. The reason for the lower

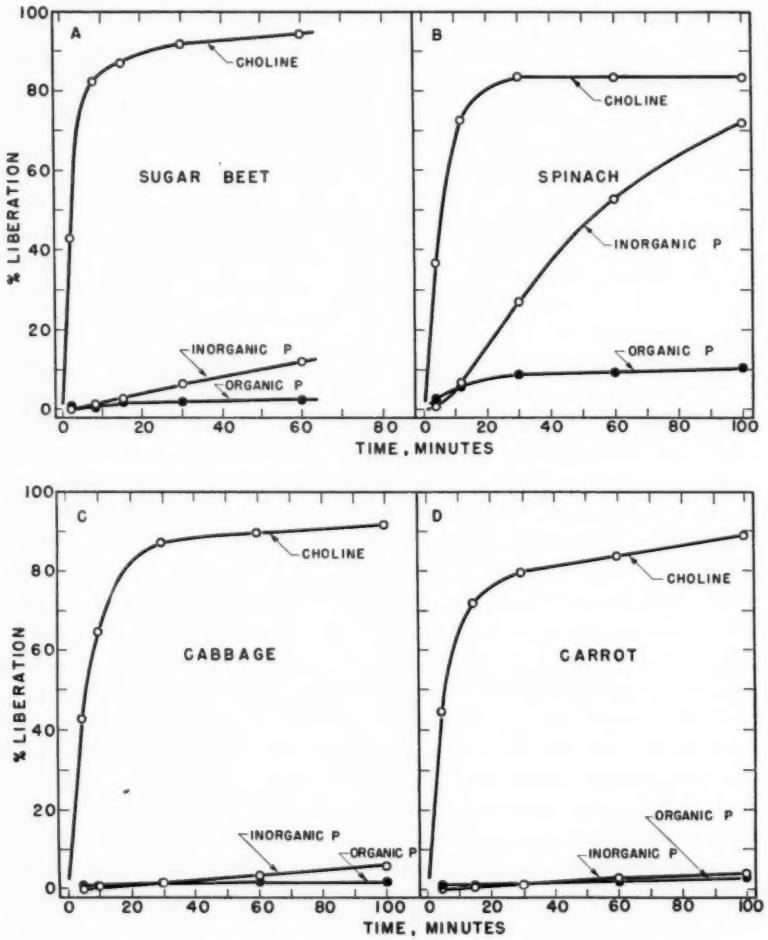


FIG. 1. Kinetics of choline, inorganic P, and water-soluble organic P liberation. Reaction carried out at indicated pH's with the following amounts of plastids (mgm.) and lecithin (μmoles) respectively: (*A*) sugar beet, 124, 32.9, (pH 4.7); (*B*) spinach, 108, 31.8, (pH 4.7); (*C*) cabbage, 128, 31.0, (pH 5.5); (*D*) carrot, 80, 31.1, (pH 5.8). Results expressed as percentage of available choline and P, and corrected for blanks without substrate, and for zero time blanks (0% choline and inorganic P, 0.9% water-soluble organic P).

choline liberation with spinach will be discussed later. The values of the first order reaction constants (k) calculated for the points on all four "choline" curves were found to be fairly constant up to about 70% liberation but decreased rapidly thereafter (Table I). The enzymatic liberation of choline from lecithin would thus appear to be a first order reaction in the early stages.

Liberation of inorganic P with plastids from all four species was relatively much slower than that of choline and was preceded by a lag period (Table I). For sugar beet, cabbage, and carrot plastids, the inorganic P liberation curves appeared to be linear with time over the periods studied (Fig. 1, Table I). For spinach chloroplasts, the curve was sigmoid in form, with a maximum slope of 1.1%/min. Furthermore, spinach chloroplasts liberated more than 70% of the P available from the lecithin as inorganic P within 100 min., while the other plastids liberated only 5 to 12% over the periods studied.

TABLE I

RATES OF CHOLINE AND INORGANIC PHOSPHATE LIBERATION[a]

Source of plastids	Time, min.	First order reaction constant for choline liberation, k,[b] min.$^{-1}$	Rate of inorganic P liberation,[c] %P/min.
Spinach	4	0.11	0.18
	12	0.11	0.76
	30	0.06	1.12
	60	—	0.87
	100	—	0.48
Sugar beet	2	0.28	0.00
	8	0.22	0.20
	15	0.14	0.20
	30	0.08	0.26
	60	—	0.18
Cabbage	5	0.11	0.00
	10	0.10	0.07
	30	0.07	0.06
	60	0.04	0.05
	100	—	0.06
Carrot root	5	0.12	0.00
	15	0.08	0.05
	30	0.05	0.04
	60	0.03	0.05
	100	—	0.03

[a] *Experimental details given in caption of Fig. 1.*

[b] $k = \frac{2.3}{t(min.)} \times \log \left[\frac{100}{100 - \%\text{ choline liberation}}\right]$.

[c] $Rate = \frac{\Delta\%\ inorganic\ P\ liberation}{\Delta t\ (min.)}$, *where* Δ*'s are increments between successive points, starting from time zero.*

Liberation of water-soluble organic P by spinach chloroplasts was not preceded by a lag period and reached 10% after 30 min. (Fig. 1*B*). With sugar beet, cabbage, and carrot plastids, water-soluble organic P was released more slowly and amounted to 2–4% of the available P, during the periods studied.

Effect of pH on Liberation of Choline, Inorganic Phosphate, and Water-soluble Organic Phosphate

The effect of pH on the liberation of choline, inorganic P, and water-soluble organic P from lecithin by spinach chloroplasts is shown in Fig. 2. The curves for the liberation of inorganic P and organic P are not true pH–activity curves, since the amount of substrate(s) available for these reactions at each pH is determined by the choline-liberating reaction. The curve for inorganic P paralleled that of choline, both curves having a maximum at pH 5.0. The curve for organic P, however, showed a maximum at pH 6.3. These results, together with those of the time–course studies (Fig. 1*B*), indicate that release of inorganic P is dependent on the prior release of choline, while liberation of water-soluble organic P is not, and probably proceeds directly from lecithin.

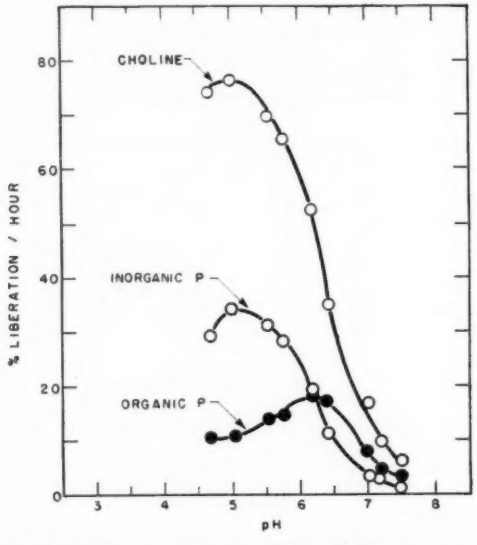


FIG. 2. Effect of pH on liberation of choline, inorganic P, and water-soluble organic P. Reaction mixtures contained 73 mgm. spinach chloroplasts, 32.4 μmoles lecithin, acetate buffers (0.1 *M* final conc.) for pH's 4.7–6.2, and tris(hydroxymethyl)-aminomethane buffers (0.075 *M* final conc.) for pH's 6.2–7.5 (12).

Distribution and Recoveries of Phosphorus and Choline After Enzymatic Hydrolysis of Lecithin

Since the enzymatic reaction mixtures consisted of aqueous, ether, and insoluble plastid phases, the distribution of choline and phosphorus among

these phases was examined. The reaction mixture of buffer, lecithin, and plastids (Table II), after incubation in the presence of ether, was acidified with trichloracetic acid and extracted thoroughly with ether, the ether extracts being washed with water. The remainder of the mixture was freed from traces of ether by stirring at 50° and was filtered with suction over Celite. The filter residue was washed with the water-washings of the ether extract, and was then extracted repeatedly with hot methanol (55°). The clear aqueous, ether, and methanol solutions were analyzed for choline (free and esterified) and total P, inorganic P, and GP P. A blank with substrate omitted was treated in the same way and the values were subtracted from those obtained with the substrate.

Recovery of P with plastids of each species was complete (98–100%), but choline recovery was significantly lower, especially with spinach chloroplasts (Table II). The low choline recoveries could have been due to the water-soluble organic P consisting mostly of a choline-containing phosphate ester, which does not form a water-insoluble reineckate. Both GPC and PC meet these requirements. As will be described below, the water-soluble organic P

TABLE II

DISTRIBUTION AND RECOVERY OF CHOLINE AND PHOSPHATE AFTER ENZYMATIC HYDROLYSIS OF LECITHIN BY PLASTIDS

For plastids of each species, reaction was carried out in six tubes; values for plastids (mgm./tube), substrate (μmoles/tube), pH, and incubation time (min.), respectively: spinach, 120, 32.2, 4.7, and 30; sugar beet, 120, 32.3, 4.7, and 30; cabbage, 133, 31.4, 5.5, and 60; carrot, 87, 32.5, 5.8, and 100; contents of six tubes combined and treated as in text.

	Distribution of available P, %						
	Aqueous				Ether	Residue[b]	
Source of plastids	Inorganic	Total organic	GP	PC[a]	Total organic	Total organic	Recovery of P, %
Spinach	21.6	12.4	2.5	9.9	50.5	15.0	99.5
Sugar beet	6.3	0.5	0.0	0.5	79.5	11.5	97.8
Cabbage	1.6	0.6	0.0	0.6	83.2	12.3	97.7
Carrot	2.6	3.7	1.3	2.4	83.1	10.5	99.9

	Distribution of available choline, %			Recovery of choline, %	
Source of plastids	Aqueous, free	Ether, bound	Residue,[b] bound	Found	Corrected for PC choline[a]
Spinach	78.8	0	11.0	89.8	99.7
Sugar beet	89.8	0	6.4	96.2	96.7
Cabbage	88.8	0	7.2	96.0	96.6
Carrot	90.2	0	7.3	97.5	99.9

[a] *% PC P = % total water-soluble organic P minus % GP P.*
= % PC choline.

[b] *Values are for methanol extract of insoluble residue.*

formed with spinach chloroplasts has been identified as PC. When the amounts of choline derived from the PC (water-soluble organic P minus GP P, if present) are added to the recoveries of choline, the corrected values agree well with the recoveries of P (Table II). The formation of a considerable amount of PC with spinach chloroplasts now explains the apparent lower choline liberation mentioned above (Fig. 1*B*).

The ether-soluble parts of the enzymatic hydrolyzates contained no choline and, except for spinach, about 80% of the available P (Table II). The ether phases are thus free from unreacted lecithin and should contain phosphatidic acid, whose isolation and identification are described below.

The insoluble residues, after exhaustive extraction with ether, still contained phospholipid material which could be extracted with hot methanol. These extracts contained 10–15% of the available P and 6–11% of the available choline (Table II). Since the choline/P ratios were less than unity (0.56–0.73), a mixture of choline-containing and non-choline-containing phospholipids must have been present in the methanol extracts. Preliminary evidence indicates that the methanol-extractable material also contains protein. These findings suggest that some of the lecithin and phosphatidic acid may have become associated with the plastids as a "lipoprotein complex".

Identification of Water-soluble Organic Phosphate

The aqueous parts of the enzymatic hydrolyzates described in the previous section were each concentrated to a small volume (3–4 ml.) and a portion was treated with 1 *M* lead acetate, centrifuged to remove lead salts, and freed from lead ions with hydrogen sulphide. Both the untreated and the lead acetate-treated portions were then subjected to paper chromatography. Blanks with substrate omitted were similarly treated and chromatographed simultaneously. Authentic samples of PC, GPC, GP, lysolecithin, and inorganic orthophosphate (all the possible water-soluble phosphorus-containing products of lecithin hydrolysis) were used as reference compounds.

The hydrolyzate from spinach chloroplasts gave two spots on the chromatogram, one corresponding to PC and the other to inorganic-P (Table III). Since GPC (R_p, 105) ran just ahead of inorganic P, it might also have been present, but masked by the orthophosphate spot. However, this possibility was eliminated when only the PC spot appeared on the chromatogram of the hydrolyzate treated with lead acetate, which removes inorganic P but not GPC or PC. The small amounts of GP present in the hydrolyzates from spinach or carrot and of organic P in those from sugar beet, carrot, or cabbage (Table II) could not be detected by the chromatographic technique used.

Identification of Ether-soluble Phosphate

As stated above, the ether extracts of the enzymatic hydrolyzates were free from unreacted lecithin and were expected to contain phosphatidic acid. When the ether extracts from the cabbage chloroplast or carrot chromoplast hydrolyzates were concentrated, diluted with ethanol, and neutralized with alcoholic sodium hydroxide, 71% and 83% respectively (blank corrected) of

TABLE III

PAPER CHROMATOGRAPHIC IDENTIFICATION OF WATER-SOLUBLE PHOSPHATES FORMED IN ENZYMATIC DEGRADATION OF LECITHIN BY SPINACH CHLOROPLASTS[a]

Material applied	Average R_P values[b]	
	Spot (1)	Spot (2)
Aqueous phase of hydrolyzate	126 ± 0	101 ± 0
Blank (no substrate)	None	101 ± 2
Aqueous phase after lead acetate treatment	126 ± 3	None
Blank after lead acetate treatment	None	None
Phosphorylcholine	123 ± 1	
L-α-Glycerylphosphorylcholine	105 ± 3	
L-α-Glycerophosphoric acid	82 ± 3	
Egg lysolecithin	267	
Inorganic orthophosphate	100	

[a] *Composition of enzymatic reaction mixture given in Table II.*
[b] *Recorded values are means (with mean deviations) of the results of two to four runs.*

the P present in the ether extracts could be recovered in the resulting sodium salt precipitate. The precipitate obtained from the cabbage chloroplast hydrolyzate, however, was contaminated with chlorophyll pigments which could not be easily removed.

The isolation of phosphatidic acid in a fairly uncontaminated state was finally achieved, with carrot chromoplasts as enzyme material, in the following way:

A suspension of 301 mgm. of lecithin (containing 403 μmoles P and choline) in 30 ml. of 0.2 *M* acetate buffer (pH 5.7) was mixed with 30 ml. of carrot root chromoplast suspension (containing 960 mgm. of plastids) and the mixture was shaken with 30 ml. of diethyl ether and incubated for 100 min. at 25° C. The mixture was acidified with 12 ml. of 1 *N* perchloric acid, filtered over a bed of Celite, and the filter residue was washed repeatedly with ether until free from pigment. The ether phase of the filtrate (150 ml.) was separated, washed several times with water, concentrated *in vacuo* (nitrogen stream) to 5 ml., and diluted with 20 ml. of 99% ethanol. After cooling to 5° C., the solution was cleared by centrifugation and neutralized by the dropwise addition of 3.0 ml. of 0.5 *N* alcoholic sodium hydroxide. The flocculent, sticky precipitate was collected by centrifugation and washed with ethanol–ether (4 : 1) and with 99% ethanol. The sodium salt was precipitated twice from its solution in diethyl ether (4 ml.) by addition of two volumes of acetone,

washed several times with acetone, and dried *in vacuo*. The dried sodium salt had a slight yellow color and weighed 294 mgm. (containing 390 μmoles P). To obtain the free (phosphatidic) acid, a solution of the sodium salt (270 mgm.) in ether (50 ml.) was shaken with several portions of 0.3 *N* hydrochloric acid followed by several portions of water, dried over sodium sulphate, and concentrated in a stream of nitrogen to a small volume. This was diluted with methanol (2 ml.) and the solution was cleared by centrifugation and concentrated *in vacuo* to a yellowish oil weighing 254 mgm.

TABLE IV

ANALYSIS OF PHOSPHATIDIC ACID OBTAINED AFTER ENZYMATIC HYDROLYSIS OF EGG LECITHIN BY CARROT CHROMOPLASTS

	Free acid		Sodium salt	
	Found	Calc.[a]	Found	Calc.[b]
% P	4.42	4.42	4.12	4.16
% N	0.03	0	—	—
N/P, atomic ratio	0.01	0	—	—
Neutral equivalent	360	351	—	—
Equivalents acid/P, ratio	1.95	2.0	—	—
% Fatty acids	75.0	80.5	—	—
Neutral equivalent	286	283	—	—
Fatty acids/P, molar ratio	1.85	2.0	—	—
$\frac{\alpha\text{-GP P}}{\text{total GP P}}$, atomic ratio	0.98[c]	1.00	0.99[c]	1.00
$\frac{\text{L-}\alpha\text{-GP}}{\text{total GP}}$, molar ratio	0.97[c]	1.00	1.00[c]	1.00

[a] *Calc. for stearoyl-linoleyl-*L*-α-glycerophosphoric acid,* $C_{39}H_{75}O_8P$ (*M.W., 701*).
[b] *Calc. for disodium stearoyl-linoleyl-*L*-α-glycerophosphate,* $C_{39}H_{73}O_8PNa_2$ (*M.W., 745*).
[c] *After saponification of the substance, 85% of the P was recovered in barium glycerophosphate; α-GP P determined by periodate method;* L*-α-GP assayed with* L*-α-GP dehydrogenase.*

Analytical data for the free acid and the sodium salt (Table IV) were in good agreement with the values calculated for the free acid and sodium salt respectively of an L-α-phosphatidic acid* containing C_{18} fatty acids. The fatty acids present were not further investigated because of insufficient material.

* *Alkaline hydrolysis of phosphatidic acid yields GP without accompanying migrations of phosphate (5). Analysis of the GP thus establishes the structure (α or β) and configuration of the phosphatidic acid.*

The isolated material contained about 20% of phosphatidic acid derived from the phospholipids present in the chromoplasts. Allowing for this, and for the fact that 84% of the available choline had been released, the yield of phosphatidic acid based on the lecithin hydrolyzed was 93%.

Action of Spinach Chloroplasts on Possible Intermediates in Lecithin Hydrolysis

The action of spinach chloroplasts on phosphatidic acid, lysolecithin, GPC, GP, and PC was studied to determine whether they could take part as intermediates in the observed liberations of choline, inorganic P, or water-soluble organic P from lecithin.

No significant release of choline or inorganic P occurred from any of the choline-containing intermediates (lysolecithin, GPC, or PC) under optimum conditions for lecithin hydrolysis (Table V). Furthermore, since GPC was completely recovered after incubation (Table V), it did not give rise to phosphorylcholine. Inorganic P was liberated from both phosphatidic acid† and

TABLE V

ACTION OF SPINACH CHLOROPLASTS ON POSSIBLE INTERMEDIATES IN THE DEGRADATION OF LECITHIN

Reaction carried out at pH 4.7 with 110 mgm. of spinach chloroplasts and indicated amount of substrate, in the presence of ether. Values are corrected for blanks without substrate.

Experiment No.	Substrate	μmoles substrate	% Liberation in 30 min.		
			Choline	Water-soluble organic P	Inorganic P
1	Egg lecithin	31.3	78	12.7	11.6
	Dipalmitoyl-L-α-glycerophosphoric acid (phosphatidic acid)	32.4	—	0.0	11.9
	Egg lysolecithin[a]	32.2	5	—[b]	0.0
2	Egg lecithin	32.6	78	10.3	20.6
	L-α-Glycerophosphoric acid[a]	31.1	—	—[c]	45.2
	L-α-Glycerylphosphorylcholine[a]	31.3	0	—[d]	0.0
	Phosphorylcholine[a]	29.3	1	—	1.9

[a] *Water-soluble compounds.*
[b] *Lysolecithin appeared to form a complex with the plastids, resulting in incomplete recovery.*
[c] *98% of the unreacted GP accounted for by periodate method.*
[d] *98% of GPC was unreacted, as estimated by periodate method.*

† *The earlier report of water-soluble organic phosphate liberation from phosphatidic acid (20) is incorrect. The synthetic material used in the previous study had decomposed partially during storage (cf. 29) to glycerophosphate and fatty acid. The sample of phosphatidic acid used in the present experiment (Table V) was freed from water-soluble organic phosphate by washing its ethereal solution with water and recrystallizing the recovered acid from acetone (m.p. 68°–69°).*

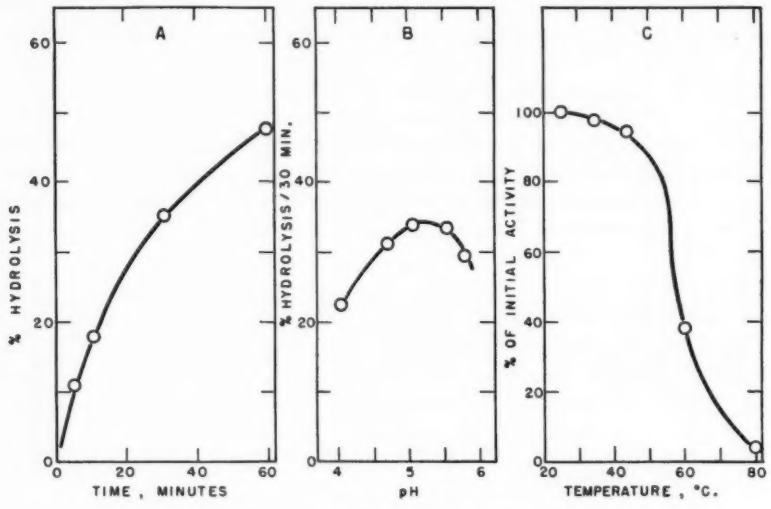


FIG. 3. L-α-Glycerophosphatase activity of spinach chloroplasts. (*A*) Time course (pH 4.7); (*B*) pH–activity relation; (*C*) Thermal inactivation: aliquots (2.5 ml.) of chloroplast suspension heated at indicated temperatures for 10 min., cooled to 25°, and their activities measured as given in text. Initial activity, 43.4% hydrolysis/30 min.

GP, the rate being the same for phosphatidic acid as for lecithin but twice as great for GP. Thus, the choline liberated from lecithin cannot arise from any of the intermediates tested; but the inorganic P could arise from phosphatidic acid and/or GP. Lack of sufficient amounts of phosphatidic acid prevented further study of the enzymatic hydrolysis of this material. Further work, however, was carried out on the hydrolysis of GP by spinach chloroplasts.

Glycerophosphatase of Spinach Chloroplasts

Pronounced glycerophosphatase activity was associated with spinach chloroplasts (Fig. 3*A*). The pH optimum was 5.2 (Fig. 3*B*). Thermal inactivation studies (Fig. 3*C*) showed that the enzyme was stable between 25° and 40° but was rapidly inactivated at higher temperatures (cf. (21)). Glycerophosphatase activity of spinach chloroplasts was found to be about 20% lower in the presence of diethyl ether than in its absence. Sodium fluoride, at 0.01 *M* concentration, completely inhibited glycerophosphatase activity. At this concentration, fluoride also completely inhibited the liberation of inorganic P from lecithin (21).

Choline Phosphokinase

The phosphorylcholine formed during the hydrolysis of lecithin by spinach chloroplasts might have arisen from phosphorylation of free choline by the

enzyme choline phosphokinase and ATP (31). However, after incubation of choline with spinach chloroplasts for one hour, either alone or in the presence of an equivalent amount of inorganic phosphate, no loss of free choline occurred and no organic phosphate appeared. Thus, under the conditions used for the enzymatic hydrolysis of lecithin, phosphorylation of choline to phosphorylcholine did not occur.

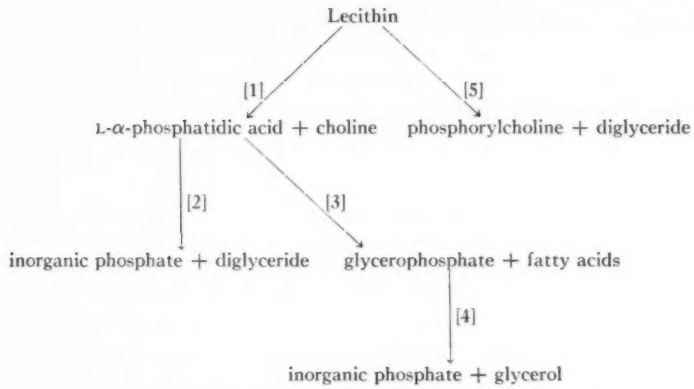
Discussion

The results of the present work with isolated plastids show that free choline arises from the direct cleavage of the choline–phosphate linkage in lecithin. Choline liberation was a rapid, first order reaction and was not preceded by a lag period (Fig. 1, Table I). Furthermore, spinach chloroplasts did not release choline from any of the possible choline-containing intermediates (Table V). Finally, phosphatidic acid, the expected product of the direct liberation of choline from lecithin, was isolated in good yield after hydrolysis of egg lecithin by cabbage or carrot plastids.

Inorganic phosphate, on the other hand, arises from the product(s) of choline liberation, as indicated by the time–course curves (Fig. 1) which display a lag phase (Table I), and by the pH–activity curve (Fig. 2). Phosphatidic acid appears to be the direct source of the inorganic P liberated from lecithin (Table V). However, since L-α-glycerophosphoric acid is also hydrolyzed (Table V), the exact sequence of reactions leading to the appearance of inorganic phosphate remains uncertain. It may arise directly from phosphatidic acid and/or from glycerophosphate formed from phosphatidic acid by loss of fatty acids. Attempts to determine free fatty acids in the hydrolyzates were unsuccessful because of the large amounts of chlorophyll degradation products present. Attempts to allow glycerophosphate to accumulate by inhibiting the glycerophosphatase with fluoride were also unsuccessful, since choline liberation, and thus the formation of phosphatidic acid, was also inhibited (21). However, the fact that small amounts of glycerophosphate were detected (Table II), together with the fact that glycerophosphate is rapidly hydrolyzed (Fig. 3*A*), would at least support the possibility of its participation in the observed inorganic phosphate liberation from lecithin.

Phosphorylcholine appears to be released directly from lecithin and independently of the liberation of choline, as indicated by the time–course curve (Fig. 1*B*) and by the pH–activity curve (Fig. 2). In the system studied, phosphorylcholine could not have been formed by hydrolysis of glycerylphosphorylcholine (Table V) nor by enzymatic phosphorylation of free choline. Its formation from lysolecithin is unlikely, since the latter is not hydrolyzed by bacterial lecithinase D (33).

The enzymatic hydrolysis of lecithin by spinach chloroplasts thus probably involves the following sequence of reactions:



With sugar beet, cabbage, or carrot plastids, the major reaction is undoubtedly reaction [1]. The small amounts of inorganic and water-soluble organic phosphate released by these plastid fractions are probably formed by reactions [2], [3], [4], and [5]. The present work therefore confirms the conclusions reached by Hanahan and Chaikoff (13) and by Acker, Diemair, and Jäger (1).

Although the enzyme which catalyzes the release of phosphorylcholine from lecithin (lecithinase D) is well known in bacterial toxins (27, 33, 9, 23, 16) the present work provides the first evidence for its existence in higher plants. The presence of glycerophosphatase in leaves is well established, but there appears to be some doubt about its location and distribution in the cell. Evidence has been reported for association of glycerophosphatase with the insoluble components of leaves (10, 11, 19) and for its presence in soluble form in the cytoplasm (10, 30). Histochemical evidence, while indicating association of glycerophosphatase with chloroplasts (32, 26), does not definitely establish its presence within the plastid (26). In the present work, glycerophosphatase appeared to be firmly associated with the spinach chloroplast fraction, but the possibility that the enzyme had been adsorbed from the cytoplasm was not excluded.

Hayaishi and Kornberg (17) confirmed the L-α-structure of egg lecithin (6) by identifying L-α-glycerophosphate among the products of lecithin hydrolysis by bacterial enzymes. Long and Maguire (25) reached the same conclusion when they obtained L-α-phosphatidic acid after phosphorylation of the diglyceride formed in the hydrolysis of lecithin with lecithinase D. The action of carrot chromoplasts now provides a method for converting lecithin directly to phosphatidic acid. The isolation of L-α-phosphatidic acid from the products of this reaction further supports the belief that naturally-occurring glycerolphosphatides possess the L-α-structure (4, 5).

Acknowledgment

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THE CONVERSION OF UROCANIC ACID TO GLUTAMIC ACID IN THE INTACT RAT[1]

By M. Kraml and L. P. Bouthillier

Abstract

Urocanic-α-C^{14} acid and imidazolepropionic-α-C^{14} acid have been synthesized and their metabolism has been studied in the intact rat. Urocanic acid has been found to be extensively catabolized and to give rise to glutamic acid. The fact that no radiocarbon could be found in tissue histidine indicates that the formation of urocanic acid from histidine is a non-reversible reaction. Imidazolepropionic acid has been found to undergo very little catabolism and thus should be excluded as a possible intermediate in the degradation of urocanic acid.

Introduction

The conversion of histidine to urocanic acid was first demonstrated by the finding that the injection of massive doses of L-histidine into rabbits and dogs resulted in the excretion of urocanic acid in the urine (20, 21). According to Edlbacher *et al.* (14, 17, 16) a liver enzyme 'histidase' catalyzed the degradation of histidine by a primary opening of the imidazole ring. Others have claimed however that the initial action of histidase is to convert histidine to urocanic acid (25, 34, 40, 41). Mehler and Tabor (25) showed that 80% of the L-histidine-2-C^{14} degraded by guinea pig liver homogenate is converted to urocanic acid. Both histidine and urocanic acid have been shown to be degraded by bacterial and liver enzymes to products, as yet incompletely characterized, which yield formic acid, ammonia, and glutamic acid on hydrolysis (4, 14, 26, 33, 32, 38, 39, 43).

The results of recent work, using C^{14}-labeled histidine, demonstrated conclusively that histidine is converted to glutamic acid both *in vitro* and *in vivo* (1, 19, 44). The distribution of isotope in the isolated glutamic acid is compatible with the theory that urocanic acid is the primary product of histidine catabolism.

However, the accumulated data concerning the metabolism of urocanic acid *in vivo* are not in favor of this compound as an intermediate in the major pathway of histidine degradation. As a matter of fact no urinary excretion of urocanic acid was observed after administration of histidine to rats (30). Furthermore, massive doses of histidine are needed to provoke urocanic acid excretion in rabbits and dogs (20, 21). Subcutaneously administered urocanic acid is largely excreted (60–90%) in the urine of rats (15). Darby and Lewis (11) have observed for other species that urocanic acid is poorly metabolized and that whenever it formed from histidine the animals exhibited signs of extreme intoxication. Celander and Berg (8) showed that urocanic acid does not promote liver glycogen regeneration in fasted rats, whereas histidine does.

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Contribution from the Department of Biochemistry, Faculty of Medicine, University of Montreal, Montreal, Quebec. Presented in part at the Annual Meeting of the Canadian Physiological Society held in Toronto, October 22, 1954.

The discrepancies existing between the results of experiments done *in vitro* and *in vivo* prompted us to investigate the metabolism of urocanic-α-C^{14} acid in the intact rat. In this paper we present evidence that urocanic acid, *in vivo*, is a direct precursor of glutamic acid.

Experimental

Synthesis of Labeled Compounds

The scheme for the synthesis of the labeled compounds is given in Fig. 1.

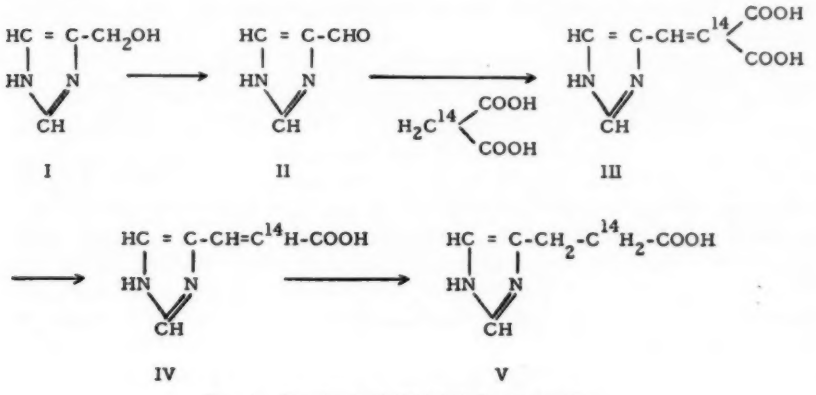

FIG. 1. Synthesis of C^{14}-labeled compounds.

Imidazole-4(5)-*formaldehyde* (II).—4(5)-Hydroxymethylimidazole hydrochloride (I) was prepared by the method of Darby, Lewis, and Totter (12). After recrystallization from hot ethanol, the product melted at 106–7° (uncorr.). The compound was converted to imidazole-4(5)-formaldehyde by oxidation with nitric acid as described by Pyman (27). The aldehyde melted at 169°–71° (uncorr.).

Imidazole-4(5)-*methylidene malonic*-α-C^{14} *acid* (III).—Imidazole-4(5)-methylidene malonic acid was prepared by the method of Akabori, Ose, and Kaneko (2). Radioactive malonic acid was obtained by the saponification of diethyl malonate-α-C^{14} (0.90 gm., 1 mc.) under reflux in an aqueous suspension of calcium hydroxide. The acid was isolated by ether extraction, at 0°–5°, of the acidified (HCl) aqueous solution. Malonic-α-C^{14} acid was condensed with imidazole-4(5)-formaldehyde (0.54 gm.) in water (10 ml.) by warming at 50° for four hours. The imidazole-4(5)-methylidene malonic acid precipitated almost quantitatively. The compound was not further purified; m.p. 206°–8° (uncorr.).

Urocanic-α-C^{14} *acid* (IV).—The crude imidazole-4(5)-methylidene malonic-α-C^{14} acid was suspended in pyridine (100 ml.) and decarboxylated by gentle refluxing for 14 hr. As the CO_2 was evolved the product slowly dissolved. Pyridine was completely removed by repeated distillation *in vacuo*. The

residue was taken up in 25 ml. of hot water and treated with Norite. Fine white needles of urocanic-α-C^{14} acid dihydrate crystallized out on cooling; m.p. 230°–1° (uncorr.). Akabori *et al.* (2) reported 231°. Calculated for $C_6H_{10}N_2O_4$: N, 16.09. Found 16.09. Ultraviolet absorption spectra taken in 0.1 *N* hydrochloric acid (pH 1.0), 0.01 *M* phosphate buffer (pH 7.4), and in distilled water (pH about 4.6) presented the same maxima as reported by Mehler and Tabor (25) for enzymatically prepared urocanic acid. Paper chromatography in four solvents (ref. Chromatography of Urine) gave only one radioactive peak coincident with the Pauly diazo-positive spot. The specific activity of radioactive urocanic acid dihydrate was 1.15×10^6 counts per minute per milligram.

Imidazolepropionic-α-C^{14} acid (V).—This was prepared by the catalytic hydrogenation, at one atmosphere hydrogen pressure, of urocanic acid (85 mgm. in 50 ml. water) in the presence of palladium catalyst (100 mgm., 5% Pd on $CaCO_3$). The hydrogenation was continued for four hours and found to be quantitative as judged by paper chromatography. Chromatograms developed in the solvent described by Mason and Berg (24) showed that urocanic acid (R_f 0.65) was completely absent. A single new Pauly diazo spot for imidazolepropionic acid (R_f 0.45) was observed. Imidazolepropionic acid dihydrate was recrystallized from water–ethanol–acetone and melted at 204°–6° (uncorr.). Its specific radioactivity was 1.14×10^6 counts per minute per milligram.

Administration of Labeled Compounds to Rats

Doses of the labeled compounds were given by intraperitoneal injection, to rats (male, Wistar strain) fasted for 12 hr. and weighing between 30–40 gm. each. Rats 1, 2, and 3 received about 10 mgm. of urocanic-α-C^{14} acid dihydrate (dissolved in 1 ml. of water; the pH was adjusted to 7.4 with sodium hydroxide). Rats 4 and 5 were given about 10 mgm. of imidazolepropionic-α-C^{14} acid dihydrate dissolved in 1 ml. of water. Each animal was placed in a glass metabolism cage and the respiratory carbon dioxide and urine were collected. After 24 hr. the rats were sacrificed with ether.

Collection of Exhaled Carbon Dioxide and Urine

The respiratory carbon dioxide was absorbed by a 10% solution of sodium hydroxide and precipitated as barium carbonate. The urine was collected and the volume was adjusted to 100 ml. with water.

Chromatography of Urine

Aliquots of the urine of Rats 1, 2, 4, and 5 were chromatographed in one dimension (ascending method) on Whatman paper No. 1. The various solvent systems employed were *n*-butanol: glacial acetic acid: water (15 : 3 : 7) (3), *n*-butanol: 95% ethanol: concentrated ammonium hydroxide (8 : 1 : 3) (29), benzene: *n*-butanol: methanol: water (1 : 1 : 2 : 1) (24), and a phenol system with water (4 : 1, in the presence of ammonia and sodium cyanide) or

saturated with borate buffer pH 9.3 (23). To reveal the presence of amino acids, the chromatograms were sprayed with a solution of 0.1% ninhydrin in *n*-butanol and heated for 15 min. at 70°. The presence of imidazole compounds was detected by spraying the chromatograms with freshly diazotized sulphanilic acid (0.5%). On dusting with powdered sodium carbonate the imidazoles give a characteristic red spot. To locate radioactive compounds on the chromatograms, segments 1 cm. wide were cut and eluted with water. The eluates were received into stainless steel cups, evaporated under an infrared lamp, and the residues were assayed for radioactivity in a gas-flow counter. Thus the loss of radioactivity due to internal absorption by the paper was avoided. The almost quantitative recovery of the radioactivity from the chromatograms illustrates the advantage of this method over the one in which the paper segments are assayed with a thin mica window Geiger tube. The latter method affords only a poor recovery of the radioactivity.

Urinary Constituents

Urocanic acid was isolated by the carrier technique from urine samples of Rats 1 and 2. To a 10 ml. aliquot of urine was added 200 mgm. of normal urocanic acid dihydrate. Heating was necessary to bring the urocanic acid into solution. The solution was treated with Norite and urocanic acid was recovered from the chilled filtrate. The product was recrystallized from hot water to constant radioactivity.

Urea was isolated as the dixanthydrol derivative from the urine of Rats 1 and 2. To a 5 ml. aliquot of urine was added 400 mgm. of urea as carrier. The solution was evaporated to dryness *in vacuo* and the residue was dissolved in 8 ml. of glacial acetic acid. An equal volume of 5% xanthydrol in methanol was added and the solution was warmed on a water bath. The rapidly formed dixanthydryl-urea precipitate was recrystallized from aqueous dioxane to constant radioactivity.

Tissue Amino Acids

The entire tissues of Rats 1, 2, and 3 were minced in a Waring blendor in the presence of 10% trichloroacetic acid. The crude proteins were removed by filtration and extracted with 5% trichloroacetic acid. The proteins were hydrolyzed in 300 ml. of 20% hydrochloric acid and certain amino acids were isolated from the hydrolyzates. After removal of tyrosine by isoelectric precipitation at pH 6.0, glutamic and aspartic acids were isolated as their barium salts. These were decomposed with sulphuric acid. Glutamic acid was crystallized as its hydrochloride while aspartic acid was purified via copper salt formation. The latter was decomposed with hydrogen sulphide to yield the free acid. Both products were recrystallized to constant radioactivity.

Histidine was isolated from the residual amino acid mixtures by precipitation at pH 7.0 as the mercury complex, according to the method of Lang (22). The mercury complex was decomposed by hydrogen sulphide and the

liberated histidine was recrystallized from water–ethanol in the presence of small amounts of arginine as hold back carrier. To remove any possible trace of radioactive arginine, the histidine was further purified by chromatography on a 4 cm. × 25 column of Dowex 50-X4 cation exchange resin (37). It was operated in the hydrogen form with 4 *N* hydrochloric acid as the eluting agent. The emergence of histidine from the column was followed by the Pauly diazo reaction performed on aliquots of each fraction spotted on filter paper.

Decarboxylation of Glutamic and Aspartic Acids

The glutamic and aspartic acids were decarboxylated by means of ninhydrin according to the method of Van Slyke *et al.* (42) and the carbon dioxide was collected as barium carbonate.

Radioactivity Measurements

Known quantities of the substances to be examined were uniformly spread on stainless steel cups (4.5 cm.^{2}) and their radioactivity measured with a Tracerlab windowless flow counter. Corrections were made for background and internal absorption.

Results and Discussion

The rates of C^{14} excretion in the respiratory carbon dioxide of Rats 1 and 2 are represented in Fig. 2. In both cases the maximum excretion occurred in the second hour period. In 24 hr., about 33 and 35% of the injected radiocarbon appeared in the expired carbon dioxide. The total excretion for 24 hr. is nearly the same as that reported for histidine-$C^{14}OOH$ (13), histidine-2-C^{14} (36) and 50% greater than the value reported by Wolf (44) for histidine-α-C^{14}. Thus urocanic acid appears to be catabolized as extensively, if not more so, than histidine itself.

The urine of Rats 1 and 2 contained about 40% of the injected radiocarbon. Isotope dilution assays revealed that 75 and 81% of the urinary radioactivity

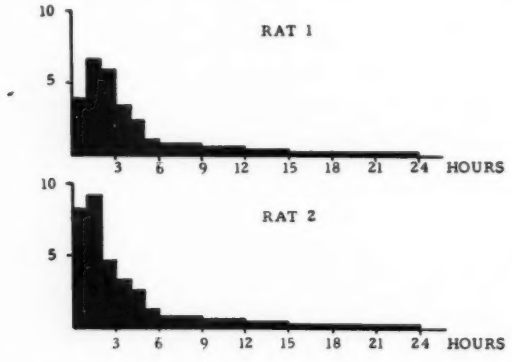

FIG. 2. Isotope excretion in respiratory CO_2 as per cent of C^{14} administered.

was accounted for by unchanged urocanic acid. This was verified by paper chromatography. A typical radiochromatogram presented in Fig. 3A shows that the urine of Rat 1 contained only one major radioactive metabolite (75–80% of the total radiocarbon in the urine). Its R_f value in the solvent system of Mason and Berg (24) is 0.51–0.54, corresponding to that of urocanic acid in urine. The remainder of the radioactivity was made up of various minor metabolites. Using the carrier technique, we have found that urea accounted for 4.0 and 2.5% of the urinary C^{14} for Rats 1 and 2, respectively.

Among the amino acids isolated from the hydrolyzates of tissue proteins (Table I) histidine was found to be devoid of radioactivity. Thus urocanic acid undergoes no reamination to histidine. This is the first direct demonstration of the irreversibility of the reaction catalyzed by histidase. Previous indications of the irreversibility of this reaction were obtained from experiments which showed that urocanic acid could not replace histidine for growth in rats (8, 9) and furthermore could not arrest weight loss in mice even in the presence of traces of L-histidine (7). Schøenheimer *et al.* (31) using N^{15} were able to show that the α-amino group of histidine was in equilibrium with the

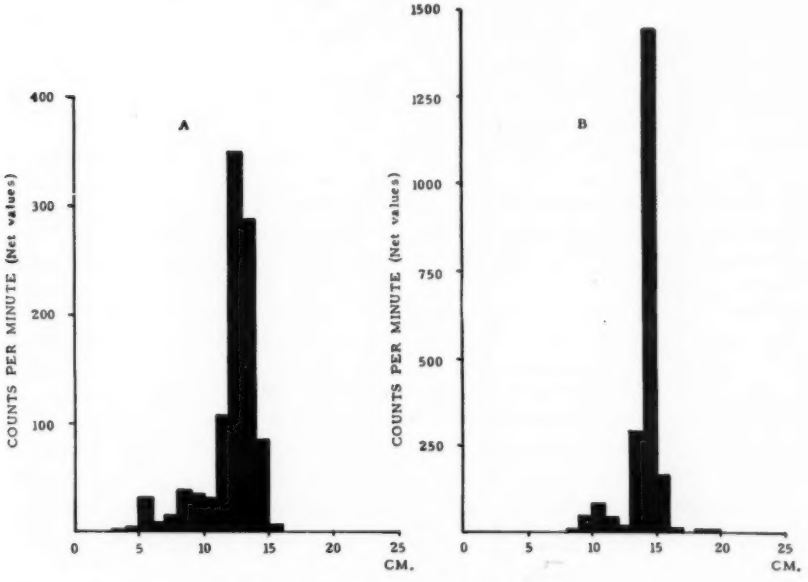

FIG. 3. Chromatography of rat urine.

(*A*). 25 λ of urine of Rat 1 (injected with urocanic acid) was chromatographed in methanol: *n*-butanol: benzene: water (24). 1 cm. segments were eluted and assayed for radioactivity. The net counts per minute are plotted against position in cm. on the chromatogram. The major peak is urocanic acid. About 95% of the C^{14} spotted at the origin was recovered from the various strips.

(*B*). 25 λ of urine of Rat 4 (injected with imidazolepropionic acid) was chromatographed in *n*-butanol- acetic acid: water (3). The major peak is imidazolepropionic acid. 98% of the C^{14} spotted at the origin was recovered.

TABLE I

RADIOACTIVITY OF TISSUE AMINO ACIDS

	Specific activity, c./min./mM.		
	Rat 1	Rat 2	Rat 3
Glutamic acid	70,500	71,400	89,400
Aspartic acid	30,200	36,700	32,500
Histidine	Nil	Nil	Nil

'labile nitrogen pool'. Evidently this could only occur via imidazolepyruvic acid and the reversible transamination reactions and not by reamination of urocanic acid.

Tissue glutamic and aspartic acids were found to be highly radioactive. On the average, the glutamic acid contained a little more than twice the amount of C^{14} present in the aspartic acid. The specific activity of glutamic acid was sufficiently high to warrant it being a product of urocanic acid catabolism. Further significance is given to the data when we take into account that only 6 and 7% of the total C^{14} present in the glutamic acid, isolated from tissue proteins of Rats 1 and 3 (Table II), was liberated by ninhydrin treatment (α-COOH). This is accounted for by carboxylation reactions. More than 90% of the isotope was contained in the remaining four carbons of the molecule and this is explainable only on the basis of a direct formation of glutamic acid from urocanic acid. This is the first demonstration that urocanic acid is catabolized to glutamic acid in the intact rat.

TABLE II

DISTRIBUTION OF C^{14} IN GLUTAMIC AND ASPARTIC ACIDS*

	Glutamic acid			Aspartic acid		
Rat No.	Specific activity, c./min./mM.	Specific activity of ninhydrin-liberated CO_2 (alpha-COOH), c./min./mM.	% C^{14} in alpha-COOH	Specific activity, c./min./mM.	Specific activity of ninhydrin-liberated CO_2 (both COOH), c./min./mM.	% C^{14} in both COOH
1	6480	435	7.0	3240	445	27.4
3	4950	290	5.9	3330	455	27.2

* *The samples of tissue glutamic and aspartic acids were mixed with normal amino acids before being decarboxylated by ninhydrin.*

The carboxyl carbons of aspartic acid contained about 27% of the radioactivity of the molecule. Although part of this radioactivity was introduced by carboxylation reactions, most of it was due to the reversible reactions and recycling processes operative in the tricarboxcylic acid cycle. It is interesting to note that the distribution of the isotope in the glutamic and aspartic acids parallels closely the values reported for these amino acids by Wolf (44) who had injected rats with histidine-α-C^{14}. Thus it is logical to assume that urocanic acid and histidine are metabolized through a common route.

Since urocanic acid is degraded to glutamic acid which is glycogenic, it is surprising that Celander and Berg (8) found that urocanic acid is not a glycogen former in the fasted rat. In our opinion this result is not sufficient to exclude urocanic acid as the primary intermediate in the conversion of histidine to glutamate. A somewhat analogous situation exists with regard to L-lysine. Although no one has been able to demonstrate that this amino acid promotes liver glycogen regeneration (6, 10, 35), recent isotope experiments have shown it to be degraded to glycogenic catabolites (28). To demonstrate liver glycogen deposition, large doses of the test substance are usually administered to fasted animals. In the case of urocanic acid, the administration of large doses would lead in all probability to its rapid and extensive elimination in the urine (15). This might explain why Celander and Berg were unable to demonstrate liver glycogen regeneration from urocanic acid. L-Histidine, which they showed to be unquestionably glycogenic, is not subject to urinary excretion, even when large doses are administered (18).

To ascertain if imidazolepropionic acid is an intermediate in the conversion of urocanic acid to glutamate, Rats 4 and 5 were injected with imidazolepropionic acid labeled with C^{14} in the α-position. The excretion of radiocarbon in the 24 hr. respiratory carbon dioxide amounted to less than one per cent of the dose administered. Thus imidazolepropionic acid undergoes very little degradation to carbon dioxide. The 24 hr. urine of Rats 4 and 5 contained 91 and 96% of the injected radiocarbon, respectively. Paper chromatography (Fig. 3B), and isotopic assays of the eluates of 1 cm. segments of the chromatograms showed that imidazolepropionic acid accounted for more than 90% of the urinary radiocarbon for each rat. It is interesting to note that these results compare with those obtained with imidazoleacetic acid in the intact rat (5), inasmuch as both undergo little oxidative degradation to carbon dioxide and are largely excreted as such in the urine.

A minor metabolite (Fig. 3B) detected in both urine samples accounts for 8 and 5% of the urinary radioactivity. Its R_f value in *n*-butanol: acetic acid: water is 0.42–0.46; in *n*-butanol: ethanol: ammonia, 0.14–0.18. This metabolite is not imidazoleacetic acid as it does not give a positive Pauly diazo test, even when the amount on the chromatograms is greatly increased. The absence of significant radioactivity in the respiratory carbon dioxide indicates that the imidazolepropionic acid molecule undergoes little if any scission and therefore the unknown metabolite may well be a conjugate.

Work is actually in progress on the complete characterization of this compound. In view of the fact that imidazolepropionic acid is not degraded in the intact rat, this compound is obviously not an intermediate in the conversion of urocanic acid to glutamic acid.

Acknowledgments

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RELATIONSHIP BETWEEN DOSE AND RESPONSE IN POSTHISTAMINIC PLASMA PEPSINOGEN IN DOGS[1]

By K. Kowalewski and S. T. Norvell, Jr.

Abstract

The effect of various doses of histamine dihydrochloride on the plasma pepsinogen in dogs was investigated. The animals were studied in acute experiments under sodium pentobarbital (Nembutal) anesthesia. They were protected against the systemic effects of histamine by the antihistaminic drug promethazine HCl (Phenergan). Sodium pentobarbital anesthesia alone or anesthesia and antihistaminic drug together did not result in the rise of plasma pepsinogen. Plasma pepsinogen was increased in the dogs treated with histamine and the degree of elevation of this enzyme varied directly according to the dose of histamine administered.

Introduction

The importance of dosage in the study of the posthistaminic increase in gastric free acid was discussed previously. A direct relationship seems to exist between the dose of histamine and the gastric secretory response (10, 6).

The same may be true concerning the endocrine activity of the stomach stimulated by histamine.

It is well established that the endocrine function of the gastric mucosa may be measured by the determination of uropepsin and plasma pepsinogen (1,13, 14, 15). A more detailed discussion on the clinical importance of uropepsin and pepsinogen and its relationship to some endocrine glands may be found in several reports (2, 3, 4, 7, 11, 15) and will not be repeated in this paper.

The effect of histamine on the gastric endocrine function was studied previously in guinea pigs. A significant increase of plasma pepsinogen was found in all animals injected with a dose of histamine sufficient to induce gastric ulcers (7, 11). We were unable to follow the posthistaminic changes in an animal for a longer period because the size of a guinea pig does not permit the repeated sampling of the blood. For this type of experiment the use of dogs seemed to be more satisfactory.

The present study is concerned with the relationship of the dose of histamine to the degree of change in the plasma pepsinogen in dogs.

Experimental

Twenty-four mongrel dogs of both sexes, fed on commercial purina dog chow and having a weight range of 7 to 21 kgm. were used in this experiment.

Six dogs of both sexes were used in a preliminary study to evaluate basic values of plasma pepsinogen in this laboratory.

Venous blood was obtained, without anesthesia, at least six hours after feeding and twice per month.

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Contribution from McEachern Cancer Research Laboratory and the Department of Surgery, University of Alberta, Edmonton, Alberta.

The remaining 18 male dogs were studied after the fasting period of at least 24 hr., and under the sodium pentobarbital anesthesia (35 mgm./kgm. body weight, intravenously). Anesthesia was used in this experiment to facilitate well timed blood sampling.

Twelve dogs used for the study of posthistaminic plasma pepsinogen were protected against posthistaminic systemic effects by promethazine HCl (5, 8, 9, 7) (Phenergan, Poulenc). It was reasonable to consider both the anesthetic and the antihistaminic as the variables in this experiment and as possible depressants or stimulants (12) of gastric activity.

The effect of antihistaminic on plasma pepsinogen in anesthetized dogs was studied in six animals. They were anesthetized with sodium pentobarbital and the blood was drawn shortly after administration of the anesthetic. Then promethazine HCl (5 mgm./kgm. body weight) was given intramuscularly and blood specimens were taken 90 and 180 min. later.

Two experiments were done on each dog from the group of 12 reserved for the study of the posthistaminic reaction.

In the first experiment the effect of anesthesia alone on plasma pepsinogen was investigated. The animals were anesthetized, as described previously, and blood was obtained immediately after anesthesia and in 90 and 180 min.

In the second experiment performed three to four weeks later, the same method of anesthesia was used. Immediately after the first sampling of blood, each dog was given antihistaminic followed in 30 min. by a subcutaneous injection of histamine dihydrochloride (Imido, Roche).

The first three animals each received 2.5 mgm. of antihistaminic and 2.0 mgm. of histamine per kgm. of body weight. The second three dogs were given 5.0 mgm. of antihistaminic and 4.0 mgm. of histamine per kgm., and the remaining six animals were each treated with 5.0 mgm. of antihistaminic and 5.0 mgm. of histamine per kgm. In all cases blood specimens were obtained 90 to 180 min. after administration of histamine.

Plasma pepsinogen was determined according to the procedure of Mirsky, but the plasma digestion of the substrate was carried out for 20 hr. and not 24 hr. as in Mirsky's technique (14).

All results are reported in terms of the amount of "tyrosine" released by the proteolytic action of a milliliter of plasma.

Results

Table I presents the results of the study of plasma pepsinogen in normal fasting dogs. The range of values of enzyme (48 determinations) is from 114 to 215 micrograms per ml. of plasma.

Table II shows the values of plasma pepsinogen in anesthetized dogs given promethazine HCl (5 mgm./kgm.). As it has been reported that the gastric exocrine secretion in guinea pigs may be increased when large doses of this antihistaminic are given (12), the possibility of effect of this antihistaminic on endocrine gastric function was considered, but in our experimental conditions it did not provoke any significant rise in plasma pepsinogen.

TABLE I

PLASMA PEPSINOGEN DETERMINED IN SIX NORMAL FASTING DOGS (TWICE PER WEEK) FOR ONE MONTH. ENZYME EXPRESSED IN MICROGRAMS OF TYROSINE RELEASED PER ML. OF PLASMA

	Dogs					
Weeks	1	2	3	4	5	6
1	203 180	167 166	153 215	160 154	114 144	167 148
2	189 185	184 191	184 157	151 162	120 152	175 171
3	193 172	186 174	182 169	153 126	144 160	122 126
4	179 203	160 201	160 201	156 171	156 203	124 120
Average	188	178	177	151	149	145
Range	172–203	160–201	160–215	126–171	114–203	120–175

TABLE II

PLASMA PEPSINOGEN IN DOGS ANESTHETIZED WITH SODIUM PENTOBARBITAL AND GIVEN PROMETHAZINE HCl (5 MGM./KGM. BODY WT.). SAMPLES TAKEN IMMEDIATELY AFTER ANESTHETIC AND 90 AND 180 MIN. AFTER ANTIHISTAMINIC. ENZYME EXPRESSED IN MICROGRAMS OF TYROSINE RELEASED PER ML. OF PLASMA

No. of animal	0 time	90 min.	180 min
7	185	154	136
8	149	150	147
9	122	150	141
10	213	168	149
11	140	147	139
12	146	124	112

Table III summarizes two experiments. In the first experiment (A) the effect of anesthesia alone on the plasma pepsinogen was studied. It may be noted that anesthesia does not stimulate the endocrine activity of the stomach. Slight reduction of plasma pepsinogen was observed in the majority of animals over a three hour experimental period.

The results of study of posthistaminic plasma pepsinogen (Experiment B) are presented in the second part of Table III. The column marked "0 time" refers to specimens obtained after anesthesia, but before the injection of

TABLE III

EFFECT OF ANESTHESIA ALONE (A) AND OF HISTAMINE IN ANESTHETIZED AND PROMETHAZINE HCl TREATED DOGS (B) ON PLASMA PEPSINOGEN. BLOOD TAKEN BEFORE THE INJECTION (TIME 0), 90, AND 180 MIN. AFTER THE INJECTION OF ANESTHETIC (A) OR HISTAMINE (B). ENZYME EXPRESSED IN MICROGRAMS OF TYROSINE RELEASED PER ML. OF PLASMA

	A				B			
	Anesthesia alone				Anesthesia, antihistaminic, and histamine			
No. of animal	0 time	90 min.	180 min.	Histamine, mgm./kgm. body wt.	0 time	90 min.	180 min.	% of rise, three hours after histamine
13	146	140	120		140	220	242	72
14	236	246	180	2.0	200	264	330	65
15	254	264	218		244	298	322	32
16	162	152	168		180	372	404	124
17	212	190	140	4.0	280	416	486	74
18	260	180	152		270	420	476	76
19	154	134	157		142	222	449	216
20	155	154	131		168	352	543	223
21	144	137	148		164	347	422	157
22	134	128	122	5.0	140	175	340	142
23	150	185	142		130	200	340	161
24	180	177	157		147	387	450	206

antihistaminic. The columns marked "90" and "180 min." refer to the samplings obtained that long after administration of histamine. The first three dogs treated with 2.0 mgm. of histamine/kgm. showed from 32% to 72% increase in plasma pepsinogen three hours after the histaminic stimulation, as compared with the initial values. The next three dogs which received 4.0 mgm. of histamine/kgm. responded by an increase from 74% to 124%, and the six dogs treated with 5.0 mgm. of histamine/kgm. showed an increase in plasma pepsinogen from 142% to 223% in the samples taken three hours after the injection of histamine, as compared with the initial values.

Fig. 1 summarizes the results of this experiment.

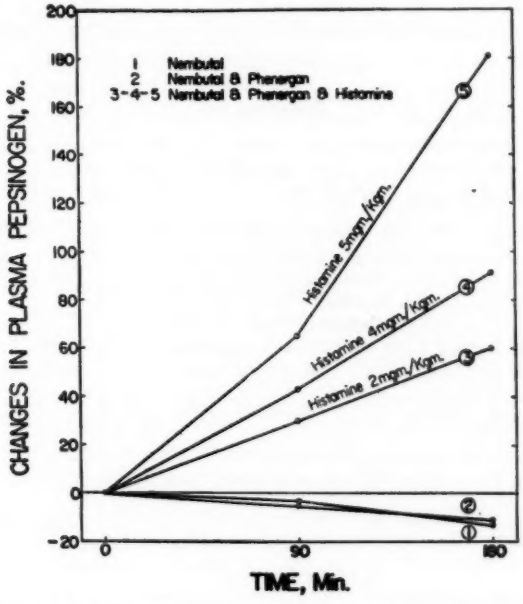

FIG. 1. Plasma pepsinogen expressed in per cent of increase of enzyme, after the injection of histamine.

Conclusions

An increase of posthistaminic plasma pepsinogen in dogs was found. The control experiments described in Tables II and III showed that anesthesia alone or anesthesia together with the antihistaminic drugs used to protect the animals has no stimulant effect on the plasma pepsinogen. The known depressive effect of barbiturates on gastric secretion may be correlated with the slight reduction of plasma pepsinogen. Under the described experimental conditions an appreciable rise in the posthistaminic level of plasma pepsinogen occurred, but the possibility can not be ruled out that a still greater rise may have been inhibited by the barbiturate.

The changes in plasma enzyme presented in Table III (Part B) are attributed to the histamine. There appears to be a direct relationship between the amount of histamine administered and the degree of increase in plasma pepsinogen values.

In the present experiment only the endocrine secretory response of the stomach to histamine was investigated. However, in recent studies on posthistaminic gastric pepsin in dogs we have noted a satisfactory correlation between the total gastric pepsin, gastric acidity, and plasma pepsinogen. This subject will be reported separately.

Acknowledgment

Histamine and Phenergan were kindly offered for this experiment respectively by Hoffman la Roche Co. and Poulenc. of Montreal.

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STUDIES WITH ALDOSTERONE[1]

By E. H. Venning, C. J. P. Giroud, I. Dyrenfurth, and J. C. Beck

Abstract

In 10 experiments, 50–98% of aldosterone added to urine could be recovered in the neutral fraction. Purification of the urinary extracts by various chromatographic procedures resulted in loss of activity. Following an intravenous administration of 600 μgm. aldosterone to an adrenalectomized patient, 13.3% was recovered in the urine in the neutral fraction. The greater part of this material was in the form of a conjugate.

Although it has been known for a long time (11, 28) that the amorphous fraction obtained from adrenal extracts contained highly active material that would maintain adrenalectomized animals and cause sodium retention (23), it was not until 1952 when Grundy, Simpson, and Tait (10) reported the isolation of a highly potent mineralocorticoid that active interest was revived in this field. The hormone has now been crystallized through the co-operation of three groups from Middlesex Hospital, London, University of Basle and Ciba Limited, Basle (18, 20), and its structure has been established as 11β, 21-dihydroxy-3-20-diketo-4-pregnen-18-al (19). Mattox *et al.* (14) also reported on the isolation and identity of this hormone. The new salt retaining hormone has been named aldosterone and many of its physiological actions are now known. They have recently been reviewed by Gaunt (8) and Wettstein (27). Aldosterone has been detected in the blood of monkeys, dogs, cattle, and man (7, 17, 16, 27). In 1950, prior to the isolation of aldosterone, Deming and Luetscher (6) reported that urinary extracts from some edematous patients with heartfailure and nephrosis showed a sodium retaining activity greater than that observed in normals and non-edematous controls. These findings have been confirmed by others (13, 26) and increased sodium retaining activity has also been demonstrated in the urine of patients with toxemia of pregnancy, cirrhosis of the liver, Cushing's disease, adrenal tumor, and malignant hypertension (4, 5, 9, 15, 22, 24, 25, 26).

The sodium retaining substance in the urine of nephrotic patients has now been identified with aldosterone (12). No attempt has been made so far to isolate and characterize the active compound present in the urine in other conditions. It is generally assumed to be aldosterone because it shows chromatographic properties similar to this hormone. Normal children and adults (1) excrete small amounts of a sodium retaining hormone. It is present in the urine as a free as well as a conjugated corticoid. Hydrolysis of the conjugate can be affected by acid or incubation with the enzyme β-glucuronidase (1, 24). Values for sodium retaining hormone excretion reported by different authors vary considerably depending upon the methods of extraction

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and hydrolysis employed. The early assays were usually carried out on the free neutral fraction and desoxycorticosterone acetate was used as the standard. More recently extracts obtained after acid or enzyme hydrolysis have been applied on paper and purified by various chromatographic procedures in an attempt to eliminate substances which may affect sodium metabolism.

Little appears to be known regarding the recovery of aldosterone from urine or the amount excreted following the administration of this hormone to humans. Through the generosity of Ciba Limited of Canada*, 1 mgm. of crystalline aldosterone was obtained. With this small amount, methods of extraction and purification of urinary extracts containing aldosterone by paper chromatographic procedures were investigated. The recovery of aldosterone in the urine following intravenous administration of this hormone to a patient was also studied. The clinical and metabolic studies on this patient will be reported in a separate paper (2).

Biological Assay

Aldosterone, or the salt retaining substance, was determined by a modification of the bio-assay of Singer and Venning (21). This method was based upon the excretion of Na^{24} in adrenalectomized adult male rats following the administration of a standard load of sodium (3.5 mgm. NaCl) containing tracer doses of Na^{24}. Desoxycorticosterone acetate was used as the standard substance. The modification consisted in omitting the radioactive sodium and measuring the total inert sodium by means of a flame photometer. The rats were maintained in a constant temperature and humidity room, 10 to 12 animals were used in each group. The sodium excretion of the test group was expressed as a percentage of the control group. This modification has the advantage of convenience only.

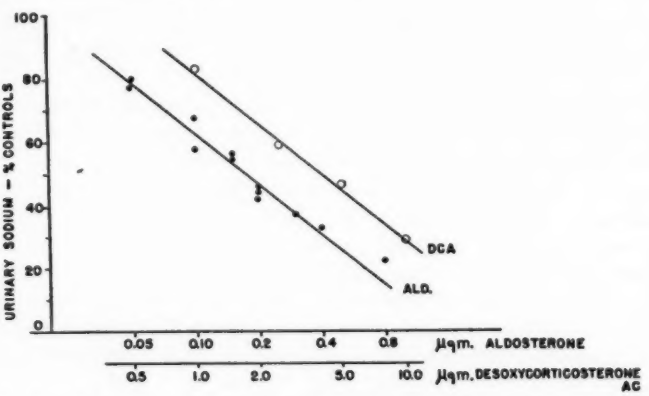

FIG. 1. Log dose – response curves for aldosterone and desoxycorticosterone acetate.

* *This material was obtained through the courtesy of Dr. Fred Wrigley and Dr. Walter Murphy of Ciba Limited, Montreal.*

Aldosterone was administered to groups of rats in amounts ranging from 0.05 μgm. to 1.6 μgm. and the effect upon sodium excretion was determined by the method described above. A linear relationship could be demonstrated between the log of the dose of aldosterone and the sodium excretion of the test animals expressed as a percentage of the control group (Fig. 1). This linear relationship holds between dose levels of 0.05 to 0.4 μgm. aldosterone. At dose levels of 0.8 and 1.6 μgm. aldosterone the linear relationship did not hold. There was a significant difference between the sodium excretion of the control group and the group receiving 0.05 μgm. aldosterone, $P \simeq 0.05$. The difference between the groups receiving 0.05 μgm. and 0.2 μgm. and those receiving 0.1 μgm. and 0.4 μgm. aldosterone was highly significant, P was <0.01. Between dose levels of 0.05 and 0.1 μgm., P was >0.1.

The index of precision for the desoxycorticosterone acetate log dose – response curve was 0.251. Two separate curves were carried out for aldosterone at an interval of one month, the values for λ being 0.227 and 0.260 respectively.

On comparing the dose – response curve obtained with aldosterone and desoxycorticosterone acetate, it was found that aldosterone was from 25 to 30 times more active than desoxycorticosterone acetate in causing sodium retention in this bio-assay.

Extraction of Urine

The extraction procedure generally used for corticoids has been applied to the extraction of the sodium retaining substance from urine. The urine is acidified to pH 1.5 with sulphuric acid and is immediately extracted three times with chloroform. For each liter of urine, 500, 250, and 250 ml. of chloroform were used. The chloroform extracts were combined and the volume was reduced to 300 ml. under reduced pressure. The chloroform was extracted twice with 20 ml. of 0.1 *N* sodium hydroxide. The alkali washings were back extracted with small volumes of chloroform. The chloroform extract was washed twice with 20 ml. water and washings were again back washed. The chloroform was evaporated to dryness under reduced pressure. The residue obtained in this manner is referred to as the free neutral fraction. That obtained after enzyme hydrolysis or prolonged acid hydrolysis is referred to as the conjugated fraction.

Effect of the pH of the Urine on the Recovery of Aldosterone

Aldosterone, 0.2 μgm. per 20 min. volume, was added to an 18-hr. volume of normal urine. The urine was divided into two equal aliquots, one was adjusted to pH 6, the other to pH 1.5 with sulphuric acid. The urines were extracted immediately with chloroform by the method described and the free neutral residues were obtained for each fraction. They were assayed at two dose levels, i.e. 20 min. volume and 40 min. volume per rat. The results were compared with the sodium retaining activity of the control urines. The

recovery of aldosterone ranged from 88% to 98% (Table I). Acidification of the urine had no effect on the recovery of added aldosterone, no apparent destruction could be detected.

TABLE I

EFFECT OF pH OF URINE ON RECOVERY OF ALDOSTERONE

pH of urine	Steroid added per volume dose, μgm.	Recovery, %
6.0	0.2	90
1.5	0.2	88
6.0	0.4	98
1.5	0.4	98

Additional Recovery Experiments

Aldosterone was added in varying amounts per volume dose to urines obtained from three other normal individuals. The urine was not acidified and the recovery of this steroid was determined in the free neutral fraction. The results are listed in Table II. In these six studies the recoveries varied from 50 to 87% of the added steroid. In all, 10 experiments were carried out, the mean recovery of added aldosterone being 81%.

TABLE II

ADDITIONAL STUDIES ON THE RECOVERY OF ALDOSTERONE FROM NORMAL URINE

	Aldosterone added per volume dose, μgm.	Recovery, %
1	0.1	72
2	0.1	87
3	0.2	84
4	0.4	50
5	0.4	64
6	0.4	75

Paper Chromatography

The neutral fraction obtained from urine contains a variety of steroids some of which may exert an influence on electrolyte metabolism. Further purification of the extract can be affected by means of paper chromatographic procedures. In an early communication by Grundy *et al.* (10) it was reported

that in the Zaffaroni (29) system (toluene – propylene glycol) aldosterone ran only slightly faster than cortisone and overlapping of the two steroids occurred. In the Bush C system (3) (toluene–ethylacetate–methanol–water) Simpson *et al.* (17) found that aldosterone runs with hydrocortisone, whereas in the Bush B-5 system (benzene–methanol–water) aldosterone runs between hydrocortisone and cortisone. The recovery of aldosterone was studied in these three systems.

Recovery of Aldosterone from Paper

In order to determine the recovery of aldosterone from paper and the effect of small amounts of cortisone and hydrocortisone, (*a*) 6 μgm. aldosterone and (*b*) a mixture containing 6 μgm. aldosterone, 10 μgm. cortisone, and 10 μgm. hydrocortisone, were applied on two paper strips 2 cm. wide and run in the Zaffaroni system (toluene – propylene glycol) for 48 hr. The whole paper strip (*a*) containing only aldosterone was eluted with methanol and assayed. A recovery of aldosterone equivalent to 110% of applied material was obtained. In the strip (*b*) containing the three corticoids, aldosterone ran with cortisone. The hydrocortisone fraction was cut away and the remaining strip was eluted with methanol. The material obtained showed the same sodium retaining activity as that obtained with aldosterone alone. In another study, 6 μgm. aldosterone and 10 μgm. hydrocortisone were applied on paper and run in the Bush B-5 system. The whole strip was eluted with methanol. The assay of this mixture showed a recovery of 100%. These studies indicate that small amounts of cortisone and hydrocortisone have no effect in this assay. The recovery of aldosterone was of the same order in the two systems.

Various solvents were tested for their efficiency in eluting aldosterone from paper. Ethanol and methanol proved to be the most satisfactory solvents for this purpose.

Urinary Extracts

1. Bush B-5 System

Aldosterone was added to a nine hour volume of urine so that each 20 min. volume contained 0.4 μgm. of this steroid. The free neutral fraction was obtained. A control specimen of this urine was extracted in a similar manner.

Aliquots of the free neutral fractions equivalent to a 280 min. volume of urine were applied on paper and run in the Bush B-5 system at 25° C. for six hours. The relative positions of the steroids on the pilot strips with reference to the zones showing ultraviolet absorption on the strips containing the urinary extracts are shown in the diagram in Fig. 2. A distinct separation of the three compounds was accomplished on pilot strip 2, no separation between aldosterone and cortisone was observed in strips 3 and 4. The strips were cut so as to include material running from the middle of the hydrocortisone band to the middle of the cortisone band (9–19.5 cm.) as indicated in the diagram. This fraction was assayed. In each case the activity of the

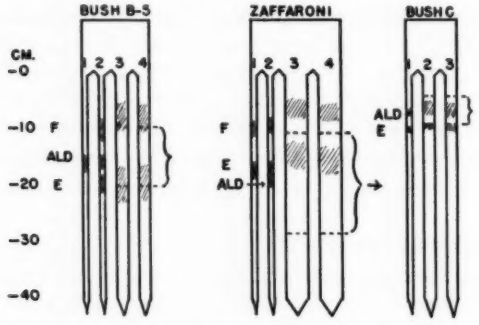

FIG. 2. Descending chromatograms of known compounds and urinary extracts. Bush B-5 system, strips 1 and 2 contain known compounds, strip 3 control urinary extract, strip 4 urinary extract with added aldosterone. Zaffaroni system, strips 1 and 2 contain known compounds, strip 3 control urinary extract, and strip 4 urinary extract with added aldosterone. Bush C system, strip 1 contains known compounds, strip 2 control urinary extract, and strip 3 urinary extract with added aldosterone. Shaded areas show ultraviolet absorption bands.

F—hydrocortisone. Ald.—aldosterone. E—cortisone.

urinary extract containing aldosterone was referred to the control urinary extract prepared in a similar way. The following outline shows the recovery of aldosterone at the various stages:

Aldosterone added to urine = 0.4 μgm. per 20 min. volume.
Neutral fraction = 0.165 μgm. per 20 min. volume.
Bush B-5 system = 0.397 μgm. per 20 min. volume.

The neutral fraction showed a poor recovery yet when this fraction was applied on paper and purified by chromatography, a better yield was obtained. The discrepancy between the two recoveries suggests that this urine may have contained substances that interfered with the assay and that purification of the extract removed them.

2. Zaffaroni and Burton System Followed by the Bush C System

In these experiments two systems of chromatography were used in an attempt to remove hydrocortisone and cortisone from the extract. Aldosterone was added to a normal urine so that 0.4 μgm. was contained in a 40 min. volume which was the dose level at which all assays were made. The neutral fraction was obtained and an aliquot representing nine hours was applied on paper and run for 48 hr. in the toluene – propylene glycol system of Zaffaroni and Burton. The relative positions of the standard substances hydrocortisone and aldosterone are shown in Fig. 2. The paper strips containing the urinary extracts were cut at 10 and 28 cm. thus eliminating the hydrocortisone zone. This material was eluted from the strip, half of it was used for a bio-assay, the remaining half was reapplied on another strip of paper and run in the Bush C System for three and one half hours at 37° C. As aldosterone runs slower than cortisone in this system, it could now be separated from the latter steroid.

The paper strip was cut at 4 and 8.5 cm. thus excluding the cortisone zone. The material was eluted and assayed. The amounts recovered in the various fractions are shown in the following outline:

Aldosterone added to urine	= 0.4	μgm. per 40 min. volume.
Neutral fraction	= 0.29	μgm. per 40 min. volume.
Zaffaroni Burton system	= 0.18	μgm. per 40 min. volume.
Bush C system	= 0.072	μgm. per 40 min. volume.

In this study there was a progressive loss of aldosterone with each purification procedure. Only 18% of the aldosterone added to the urine was recovered in the final extract.

Recovery of Aldosterone from Urine Following Intravenous Administration

Fifteen hundred milliliters of a solution containing 5% glucose and 600 μgm. aldosterone were administered in a continuous intravenous infusion over a period of 24 hr. to a bilaterally adrenalectomized patient who was being maintained on a daily dose of 15 mgm. cortisone. The urine collection was divided into three periods:

(1) A control period of 48 hr.
(2) A second period of 36 hr. collected during the intravenous infusion and 12 hr. afterwards.
(3) A further period of 36 hr.

The urine excreted during each period was acidified to pH 1.5 and extracted immediately with chloroform. This fraction is referred to as the free fraction and was assayed separately. The remaining urine was investigated for the presence of conjugated aldosterone. It has been shown by two methods of hydrolysis that increased sodium retaining activity can be obtained from urine by hydrolysis with β-glucuronidase (24) and hydrolysis with acid at room temperature (1). These two methods were investigated.

The urine was divided into two aliquots, one (E) was submitted to β-glucuronidase hydrolysis, the other (A) to acid hydrolysis. For the hydrolysis by β-glucuronidase the urine was adjusted to pH 4.8, 200 units of β-glucuronidase* were added and the mixture was incubated at 37° C. It was extracted with chloroform at 4 and again at 48 hr. The chloroform extracts were combined. For the acid hydrolysis the urine was adjusted to pH 1.5 and allowed to stand at room temperature for 48 hr. An aliquot of each of the neutral fractions obtained by these various methods was assayed.

In order to determine whether all the aldosterone had been hydrolyzed in the urine by these procedures, the acid hydrolyzed urine obtained in the second period after extraction was submitted to enzyme hydrolysis and similarly the enzyme hydrolyzed urine to a further acid hydrolysis. No further activity could be extracted when the acid hydrolyzed urine was subjected to enzyme hydrolysis. However, when the enzyme hydrolyzed urine was allowed to

* *We are greatly indebted to Warner Chilcott Laboratories for the liver β-glucuronidase, "Ketodase", used in these experiments.*

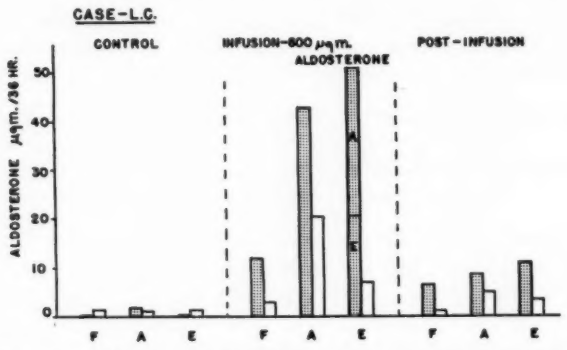

FIG. 3. Excretion of aldosterone following intravenous administration of 600 µgm. aldosterone. Each period represents 36 hr. Shaded columns represent neutral fractions; white columns represent fractions purified by chromatography.
F—free fraction. A—acid hydrolyzed fraction. E—enzyme hydrolyzed fraction.

stand for 48 hr. at pH 1.5 an additional amount of 30.2 µgm. aldosterone was obtained. The amounts of aldosterone recovered by these procedures in the neutral fraction are shown in the shaded columns in Fig. 3.

Essentially no aldosterone activity was found in the urine collected during the control period. The greatest amount was excreted during the infusion period and only small amounts were recovered in the post-infusion period. The total amount of aldosterone excreted as the free steroid in periods 2 and 3 amounted to 17.9 µgm. in the neutral fraction. The amount that was released by combined enzyme and acid hydrolysis was equivalent to 62.0 µgm. aldosterone. Thus the total amount of aldosterone excreted over the period of 72 hr. during and following the intravenous administration of 600 µgm. aldosterone was 79.9 µgm., representing a recovery of 13.3% of the administered hormone.

An aliquot of each of the various neutral fractions was applied on paper and chromatographed in the Bush B-5 System. The paper strips were cut in a manner similar to that already described for this system. The results of the assay of these fractions are represented by the white columns in Fig. 3. The total amount of aldosterone recovered, using this method of purification, was 30 µgm. Thus loss of aldosterone occurred during the chromatographic procedure.

Discussion

On account of the small amount of aldosterone available for this investigation it was only possible to carry out a limited number of experiments. In many instances no duplicate studies could be made. Therefore it is not possible with this number of data to draw any conclusions with regard to the relative merits of the different systems. It would seem from the results obtained that chloroform is a satisfactory solvent for the extraction of

aldosterone from urine and that ethanol and methanol are efficient in removing aldosterone from paper strips. When aldosterone was applied on paper either alone or with small amounts of pure corticoids and run in the Zaffaroni and the Bush B-5 systems, the elution of the whole strip gave satisfactory recoveries. The application of partition paper chromatography for the purpose of purification of urinary extracts with the elimination of certain zones on the paper gave variable results. In one study the neutral fraction gave a low result, whereas after purification by chromatography a higher recovery of aldosterone was obtained. In general, however, losses occurred during the procedure of chromatography.

When aldosterone was administered intravenously to a totally adrenalectomized patient, this substance was excreted in the urine as free and conjugated corticoid. Other metabolities of aldosterone have not yet been identified. It is evident from the study on hydrolysis that incubation with β-glucuronidase does not release all the conjugated aldosterone. Other studies carried out in our laboratory substantiate this finding. More sodium retaining activity can be obtained if a second hydrolysis with acid is carried out.

Summary

Extraction and paper partition chromatographic procedures have been investigated with reference to the recovery of crystalline aldosterone added to urine. The average recovery in the neutral fraction was 81%. Loss occurred when the neutral fraction was applied on paper and run in various chromatographic systems.

Aldosterone (600 μgm.) was administered intravenously to a bilaterally adrenalectomized patient and 13.3% was recovered in the urine. The greater part of the active mineralocorticoid was present in the urine as a conjugate. Incubation with β-glucuronidase does not release all the aldosterone from conjugation. The application of a subsequent acid hydrolysis will liberate further amounts of this sodium retaining hormone.

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THE ANAEROBIC DISSIMILATION OF D-RIBOSE-1-C^{14}, D-XYLOSE-1-C^{14}, D-XYLOSE-2-C^{14}, AND D-XYLOSE-5-C^{14} BY *AEROBACTER AEROGENES*[1]

By H. A. Altermatt[2], F. J. Simpson, and A. C. Neish

Abstract

The lactic acid produced from D-ribose-1-C^{14} or D-xylose-1-C^{14} had only a trace of isotopic carbon in the carbinol group, while the methyl carbon had 40% and carboxyl carbon 20% of the specific activity of carbon-1 of the pentose. The lactic acid from D-xylose-2-C^{14} was labelled to a slight extent in the methyl group, while the carbinol carbon and the carboxyl carbon had 40% and 20% respectively of the C^{14} concentration of carbon-2 of the sugar. D-Xylose-5-C^{14} gave lactic acid labelled mainly in the methyl carbon, which had about 60% of the specific activity of carbon-5 of the pentose. The other fermentation products (2,3-butanediol, ethanol, acetic acid, formic acid, and carbon dioxide) were labelled as if they had been formed from pyruvate with the same labelling as the lactic acid. These results offer additional support to the hypothesis whereby complete conversion of pentose to triose occurs via a heptulose.

Introduction

Recent intensive studies on the intermediates and enzymes of the hexose monophosphate shunt have stimulated interest in the methods by which pentoses are degraded by microorganisms. Lampen *et al.* demonstrated with *Lactobacillus pentosus* that the methyl group of acetic acid was derived from carbon-1 of xylose-1-C^{14}, thus supporting earlier suggestions of a cleavage of the five carbon sugars into two-carbon and three-carbon units (4, 18). Similar results have been obtained from the fermentation of D-ribose-C^{14} by *L. pentosus* (3), of L-arabinose-1-C^{14} by *Lactobacillus pentoaceticus* (28), of D-xylose-1-C^{14} by resting cells of *Fusarium lini* (7) and *Leuconostoc mesenteroides* (1), and of D-xylose and L-arabinose by *Acetobacter acetigenum* (15). The pentose, arising from the oxidative decarboxylation of glucose, is apparently cleaved in the same manner by *Leuconostoc mesenteroides* (8), but the two-carbon fragment is converted to ethanol instead of acetic acid.

Propionibacterium pentosaceum is also believed to cleave pentoses between carbons 2 and 3, but the two-carbon fragment does not pass directly to acetate as with the lactic acid bacteria, rather the two-carbon fragment passes through a series of reactions that lead to the presence of carbon-1 of the pentose in both carbons of the acetate and in the methyl and methylene carbons of propionic acid (27). Similarly with *Aerobacter aerogenes* (23) and *Escherichia coli* (6) the two-carbon fragment is not directly converted to acetate or ethanol, but is used in subsequent reactions that lead to the distribution of carbon-1 of the pentose in both the methyl and carboxyl carbons of lactate. To explain the results obtained with the dissimilation of 1-C^{14} labelled D- and L-arabinose by *A. aerogenes*, Neish and Simpson proposed that pentoses were

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[2] *National Research Council of Canada Postdoctorate Fellow 1953–1955.*

completely converted to pyruvate via a heptulose (23, 24). In the present investigation the labelling predicted by the proposed carbon skeletal changes agree with the distribution of C^{14} found in the products when ribose-1-C^{14}, xylose-1-C^{14}, xylose-2-C^{14}, and xylose-5-C^{14} were anaerobically fermented by *A. aerogenes*.

Experimental

Preparation of Labelled Compounds

D-Ribose-1-C^{14} was synthesized from D-erythrose, and D-xylose-1-C^{14} from D-threose, by the cyanohydrin reaction as described previously (21, 22). To obtain D-xylose-2-C^{14}, the D-arabinose-1-C^{14}, obtained as a by-product in the preparation of D-ribose-1-C^{14}, was reacted with sodium cyanide (14, 22) and after resolution and reduction of the products, the glucose-2-C^{14} obtained was converted to D-xylose-2-C^{14} by Sowden's method of degradation (29). The yield of D-xylose-2-C^{14} was 56% based on glucose-2-C^{14}.

To prepare D-xylose-5-C^{14} 2 mM. of L-arabinose was treated with an equimolar amount of C^{14}-labelled potassium cyanide (1 mc.) (14) to give L-gluconic and L-mannonic acids. These epimeric acids were separated and the crystalline barium L-gluconate was transformed to the δ-lactone, which was then reduced with sodium borohydride to L-glucitol-1-C^{14} (32). Treatment with furfuraldehyde in the presence of 3 *N* sulphuric acid produced 2,4-furfurylidene-*L*-glucitol (11). This was oxidized with lead tetraacetate to monofurfurylidene-D-xylose-5-C^{14} and the desired sugar released by hydrolysis. The over-all yield of D-xylose-5-C^{14}, based on the radioactive cyanide, was 11%.

Fermentations

Aerobacter aerogenes, PRL R3 (NRRL 199), was used throughout and the results from the fermentation of xylose-1-C^{14} were checked with another strain, PRL R4. The conditions for growing and harvesting the cells were the same as those described previously, except that the substrate was either ribose or xylose as required to ensure adaptation to the sugar being studied (24). The cells were suspended in 12 ml. of a sterile medium containing 2.5% $MgNH_4PO_4 \cdot 6H_2O$, 0.25% K_2SO_4, and 0.002% phenol red. Ten milliliters of the suspension was aseptically transferred to a modified Erlenmeyer flask (20). To this was added 5 ml. of a sterile solution containing 0.5 gm. of the labelled sugar, and the air in the flask was then replaced with oxygen-free nitrogen (0.9 atm.). The flasks were placed in a water bath maintained at 30° C. The pH was controlled manually by the frequent addition of 0.03 to 0.1 ml. of 1 *N* sodium hydroxide (24).

Analysis of Fermentation Products

The products were measured and isolated as before (20). 2,3-Butanediol was oxidized to acetaldehyde by periodic acid and the acetaldehyde subsequently oxidized to acetic acid by chromic acid (22). Lactic acid was oxidized

with permanganate to carbon dioxide and acetaldehyde, then the latter was converted to acetic acid. Ethanol was also oxidized to acetic acid by acid dichromate (22). After conversion to the sodium salt and drying, acetic acid was degraded by the method of Phares (25). The resulting samples were converted to carbon dioxide and the activities measured in a proportional counting apparatus (22).

Results

The products obtained from fermentation of the labelled pentoses are given in Table I. The lower yield of 2,3-butanediol and the higher yield of organic acids, particularly formic, obtained with xylose-1-C^{14}, xylose-2-C^{14}, and xylose-5-C^{14} are likely caused by a high pH. These fermentations were maintained on the pink to red side of phenol red about 7.6–8.0, whereas the others were held more closely in the transition zone (7.0–7.8). The low yield of 2,3-butanediol in these fermentations made it necessary to add a large amount of carrier. Because of this the determinations of C^{14} were considered unreliable and were not included in Table II.

TABLE I

PRODUCTS OBTAINED FROM THE ANAEROBIC DISSIMILATION OF SUGARS BY *A. aerogenes* R3 AND R4

Product	Millimoles per 100 millimoles of sugar fermented				
	Ribose-1-C^{14} R3	Xylose-1-C^{14} R4	Xylose-1-C^{14} R3	Xylose-2-C^{14} R3	Xylose-5-C^{14} R3
2,3-Butanediol	10.3	3.7	11.0	1.4	1.4
Ethanol	59.6	55.6	49.5	46.6	39.9
Acetic acid	54.6	61.0	48.1	60.4	58.8
Formic acid	59.4	72.3	47.0	73.8	69.7
Succinic acid	11.0	6.0	4.9	10.1	7.6
Lactic acid	3.48	3.3	4.4	13.5	10.6
Carbon dioxide	60.0	46.0	38.4	32.3	36.2
Fermentation time (hr.)	12.0	6.5	5.0	6.5	5.5
Sugar dissimilated (%)	100	89.1	97.0	92.0	98.6
C^{14} in cells[1]	7	9.6	5.8	6.0	6.0
Sugar carbon accounted for (%)	95.7	89.6	77.3	87.3	80.2

[1] *As per cent of added C^{14} found in cell fraction.*

The distribution of C^{14} in the fermentation products from ribose-1-C^{14} and xylose-1-C^{14} is of the same order and this is also true for xylose-1-C^{14} fermented by either strain of *A. aerogenes*. The methyl groups of 2,3-butanediol, ethanol, acetic acid, and lactic acid contain about two fifths of the radioactivity originally present in the pentose whereas the carboxyl carbon of lactic acid, formic acid, and carbon dioxide contains approximately one fifth. With xylose-2-C^{14} the carbinol carbons of ethanol and lactic acid and the carboxyl carbon of acetic acid contain the heaviest labelling. Only the methyl groups of the products contained significant amounts of C^{14} when xylose-5-C^{14} was the substrate.

TABLE II

DISTRIBUTION OF C^{14} IN FERMENTATION PRODUCTS

Product	C^{14} concentration as % of that in labelled carbon of sugar used				
	Ribose-1-C^{14} R3	Xylose-1-C^{14} R4	Xylose-1-C^{14} R3	Xylose-2-C^{14} R3	Xylose-5-C^{14} R3
2,3-Butanediol					
CH_3—	39.0	26.8*	38.8	**	**
—CHOH—	0.4	0.2*	0.3		
Ethanol					
CH_3—	41.0	40.9	31.2	0.3	60.4
—CH_2OH	0.4	0.4	0.3	37.9	0.5
Acetic acid					
CH_3—	41.5	41.0	40.1	0.3	60.7
—COOH	0.4	0.3	0.4	40.9	0.6
Lactic acid					
CH_3—	48.0*	31.1*	35.7*	0.6	57.6
—CHOH—	0.6*	0.5*	0.0*	40.6	0.6
—COOH	27.0*	18.6*	20.9*	19.8	0.7
Formic acid	25.4	21.9	23.7	19.2	0.6
Carbon dioxide	19.0	20.7	16.4	19.7	1.8
Cell carbon	6.6	10.4	2.3	4.6	5.1

* *High dilution (10 times) by carrier necessary in this determination.*
** *Insufficient butanediol for accurate measurement.*

Discussion

The results give additional support to the scheme previously advanced to explain the dissimilation of D- and L-arabinose-1-C^{14} by *A. aerogenes* (24). This scheme (Fig. 1) assumes complete conversion of the pentose by transketolase and transaldolase (or aldolase) to triose phosphate which is subsequently dissimilated via pyruvate to lactic, acetic, and formic acids, 2.3-butanediol, ethanol, and carbon dioxide.

The carbon atoms in Fig. 1 are numbered with reference to those of the pentose to facilitate following the labelled carbons. Three molecules of pentose are assumed to be converted to the equilibrium mixture of ribose-5-phosphate and ribulose-5-phosphate (9, 10, 17). Two molecules of ribulose-5-phosphate are then cleaved between carbons 2 and 3 by transketolase to produce two molecules of triose phosphate and two molecules of "active glycolaldehyde" as depicted in Fig. 1A (26). A molecule of ribose-5-phosphate, representing the third pentose molecule, acts as the acceptor for one "active glycolaldehyde" forming sedoheptulose phosphate (Fig. 1B) (13, 26). If sedoheptulose-7-phosphate is the seven-carbon intermediate, transaldolase would be expected to transfer the first three carbons of sedoheptulose to glyceraldehyde phosphate (12, 13). The hexose monophosphate formed would then be phosphorylated and cleaved to yield two molecules of triose phosphate.

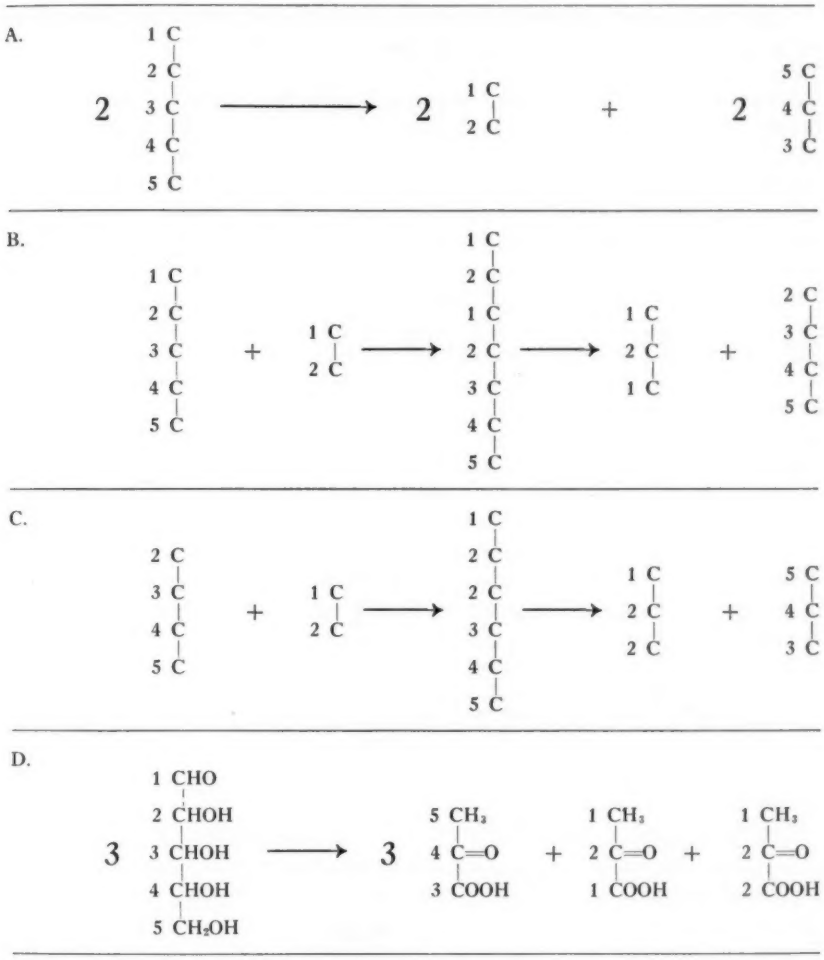

FIG. 1. Hypothetical C-skeleton changes in pentose metabolism.

On the other hand, if sedoheptulose-1,7-diphosphate is the seven-carbon intermediate the cleavage could be catalyzed by aldolase since sedoheptulose diphosphate possesses trans hydroxyls at carbons 3 and 4 and would yield dihydroxyacetone-1-phosphate (30). Either way, the result is conversion of heptulose phosphate to a molecule of triose phosphate and tetrose phosphate labelled as shown in Fig. 1B. The tetrose phosphate (D-erythrose-4-phosphate) is assumed to accept the other molecule of "active glycolaldehyde" to form fructose-6-phosphate (12, 13). Phosphorylation of this compound and its transformation to two trioses would then follow the glycolytic route (Fig. 1C).

The over-all result of these changes, as shown in Fig. 1D, is the conversion of three molecules of pentose to five molecules of pyruvate. The pyruvate from pentose-1-C^{14} would have two fifths of the activity of carbon-1 of the pentose in the methyl carbon, and one fifth in the carboxyl carbon. The methyl groups of the lactic acid, acetic acid, ethanol, and 2,3-butanediol subsequently formed from this pyruvate would contain two fifths, and the carboxyl carbon of lactic acid, formic acid, and carbon dioxide would contain one fifth of the C^{14} originally present in carbon-1 of the pentose. Xylose-2-C^{14} should give rise to pyruvate with two fifths of the activity of carbon-2 of the pentose in the carbonyl carbon, and one fifth in the carboxyl carbon. Thus the carbinol carbon of lactic acid, ethanol, and 2,3-butanediol, and the carboxyl carbon of acetic acid would contain two fifths, and the carboxyl carbon of lactic acid, formic acid, and carbon dioxide, one fifth of the activity of carbon-2 of the pentose. Since carbons 3, 4, and 5 of the pentose are converted as a unit to pyruvate, all of the C^{14} from xylose-5-C^{14} would reside in the methyl groups, but would be diluted by the methyl groups derived from carbon-1 of the pentose. Thus the activity of the methyl carbons of the fermentation products would be three fifths of carbon-5 of the sugar. The results in Table II agree with these expected values.

The fortuitous combination of two other alternate pathways might be used to explain the results obtained from the fermentation of pentose-1-C^{14}. Cleavage of pentose-1-C^{14} between carbons 3 and 4, as suggested (31), would produce methyl labelled lactic acid but would not explain the presence of C^{14} in the carboxyl group. Oxidation of carbon-1 and subsequent cleavage such as found in *Pseudomonas saccharophila* and *P. lindneri* (5, 19) would explain the presence of C^{14} in the carboxyl group but not in the methyl group of lactic acid. Such a combination, however, would not explain the distribution of C^{14} in the products obtained from the fermentation of xylose-2-C^{14} or xylose-5-C^{14}.

The proposed mechanism (Fig. 1) for the fermentation of pentoses by *A. aerogenes* is similar to that believed to function in the interconversion of monosaccharides in plants and animals (26). A modification of the scheme has been used with some success in interpreting results obtained from the fermentation of sedoheptulose by *A. aerogenes* (22). These interconversions of pentose $\rightleftharpoons$ heptulose $\rightleftharpoons$ hexose appear to be quite important in higher organisms where the interconversions may function in the transformation of the products of photosynthesis and in the complete respiration of hexoses (2,16). The occurrence of these reactions in *A. aerogenes* indicates the complex metabolic organization of this organism as compared to certain other bacteria.

Acknowledgment

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THE ANAEROBIC DISSIMILATION OF D-XYLOSE-1-C^{14}, D-XYLOSE-2-C^{14}, AND D-XYLOSE-5-C^{14} BY *LEUCONOSTOC MESENTEROIDES*[1]

By H. A. Altermatt[2], A. C. Blackwood, and A. C. Neish

Abstract

D-Xylose was dissimilated anaerobically by *Leuconostoc mesenteroides* to an equimolecular mixture of acetic and lactic acids. Xylose-1-C^{14} gave methyl-labelled acetic acid, xylose-2-C^{14} gave carboxyl-labelled acetic acid, and xylose-5-C^{14} gave methyl-labelled lactic acid. The amount of C^{14} found in any other position was less than one per cent of the total.

Introduction

Leuconostoc mesenteroides was found by DeMoss *et al.* (7, 8) to ferment glucose to an equimolecular mixture of carbon dioxide, ethanol, and lactic acid. The constant ratio of these products under varying cultural conditions and the absence of aldolase and other enzymes led these workers to suspect that a hitherto unknown glycolytic mechanism was operating in this organism. This suspicion was confirmed by studies on the fermentation of glucose-1-C^{14} and glucose-3,4-C^{14} (4, 12). The results indicated that the carbon dioxide came solely from carbon-1 of the sugar, the ethanol methyl group from carbon-2, the ethanol carbinol group from carbon-3, the lactic carboxyl group from carbon-4, the lactic carbinol group from carbon-5, and the lactic methyl group from carbon-6. We have further confirmed these findings by fermenting glucose-2-C^{14} with this organism (unpublished results); the isotopic carbon was concentrated (97.7%) in the methyl group of the ethanol as predicted.

This fermentation provides a convenient method for degradation of labelled glucose for determination of the C^{14}-distribution. It has been used in studies on the biosynthesis of glucose from glycerol (20) and from pentose phosphate (11, 13) and also in an investigation of the biosynthesis of cellulose from labelled monosaccharides in wheat plants (15). In the last-mentioned work it was desired to degrade the xylose isolated from the xylan also. A chemical method has been described (2) that should be suitable for this purpose but the convenience of the biological procedure used for degrading glucose prompted investigation of a similar method for xylose.

Studies on the fermentation of L-arabinose-1-C^{14} by *Lactobacillus pentoaceticus* (19) and on the fermentation of D-xylose-1-C^{14} by *L. pentosus* (10) have shown that these organisms give a mixture of acetic and lactic acids with the isotopic carbon concentrated in the methyl group of the acetic acid. This was explained by postulating a splitting of the pentose chain between carbon-2 and carbon-3. The two-carbon fragment then gives acetic acid with the

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National Research Council of Canada Postdoctorate Fellow, 1953–55.

methyl group coming from carbon-1 of the pentose and the carboxyl group from carbon-2. The lactic acid presumably arises from the three-carbon segment (triose phosphate) with the carboxyl group coming from carbon-3 of the pentose and the carbinol and methyl groups arising from carbon-4 and carbon-5 respectively.

Leuconostoc mesenteroides also ferments arabinose to an equimolecular mixture of acetic and lactic acids (17) so it would be expected to ferment pentoses in a manner similar to that of the lactobacilli, particularly since D-ribulose-5-phosphate is a probable intermediate in the dissimilation of glucose (5, 9). This culture was being used for the degradation of glucose and it was convenient to use it for the degradation of xylose. In order to establish that the lactic and acetic acids would be labelled as indicated above, synthetic samples of D-xylose-1-C^{14}, D-xylose-2-C^{14}, and D-xylose-5-C^{14} were fermented and the C^{14}-distribution in the products determined.

Experimental

The preparation of the labelled xyloses has been outlined in another communication (1). The wheat xylose was obtained from Dr. G. Krotkov, Queen's University, Kingston, Ontario. It was isolated from the hemicellulose of C^{14}-labelled wheat plants.

The fermentations were run anaerobically, usually under aseptic conditions, with manual pH control in modified Erlenmeyer flasks (14) with a resting cell suspension of *Leuconostoc mesenteroides* PRL L33. The culture was obtained through the courtesy of Dr. R. D. DeMoss of Johns Hopkins University and was grown on a medium suggested by DeMoss (6) containing 1% glucose, 1% yeast extract, 1% tryptone, 0.5% K_2HPO_4, and 2% salts (0.8% $MgSO_4 \cdot 7H_2O$, 0.04% $FeSO_4 \cdot 7H_2O$, 0.04% NaCl, 0.19% $MnCl_2 \cdot 4H_2O$, and 0.5% concentrated HCl). The various constituents were sterilized separately and combined at the time of inoculation. Stock cultures were carried on the same medium plus 2% agar.

Although the culture was grown under standardized conditions the cells frequently had little or no activity on either glucose or xylose, but when the cells were active they metabolized the substrate very rapidly. In an attempt to solve this problem numerous trials were made by varying growth conditions, medium, methods, and time of harvesting the cells, etc.; activity was measured with the Warburg technique where carbon dioxide evolved was recorded under anaerobic conditions. One observed phenomenon was that growth of this culture is constant and quite rapid with either glucose or xylose as the carbohydrate source, but when both glucose and xylose are added growth is faster for some unknown reason and the cells are nearly always active on either glucose or xylose substrate. For pentose fermentations the culture must be grown on a pentose substrate.

The following conditions were found to give the most reliable results. The culture was maintained on slants, stored in the cold, then transferred to broth and incubated 24 hr. at 30° C. A small amount of this inoculum (0.1%) was

added to a 500 ml. Erlenmeyer flask containing 400 ml. of medium as given above except that the carbohydrate source was 0.5% glucose plus 0.5% xylose. The culture was incubated at 28°–30° C. for 10–12 hr. without agitation. Growth was not heavy at the time of harvesting as the culture was just beginning the logarithmic growth phase. The cells were centrifuged down, washed once with phosphate buffer (0.02 *M* KH_2PO_4), recentrifuged, and resuspended in a volume of buffer equal to about one-tenth the original volume of medium. The cells were used immediately as storage under any conditions that were tried caused rapid loss of activity. A rapid preliminary trial with the Warburg technique was found useful in establishing the relative activity of the cells.

The cell suspension (5–10 ml. volume) was added to a flask containing 2 mM. of the labelled sugar (0.6–2.0 microcuries) in 5–10 ml. of water and 1 ml. of an aqueous 0.02% solution of bromcresol green (sodium salt). The flasks were sealed and flushed with nitrogen (14) and incubated at 30° C. Aseptic technique was used throughout. The pH was controlled by injection of 0.01–0.03 ml. of *N* NaOH whenever the solution became typically yellow-green. When acid formation ceased, the fermented liquor was treated as described below. With active cells a six to eight hour fermentation period was sufficient for complete sugar utilization.

The fermentation solutions were acidified, clarified with zinc hydroxide, the lactic and acetic acids recovered by ether extraction and separated by partition chromatography on a silicic acid column as described previously (16). The lactic acid was converted to carbon dioxide and acetic acid (16), and the acetic acid was degraded by the method of Phares (18). Each fermentation thus gave five different carbon dioxide samples for C^{14} assay, representing the acetic acid methyl and carboxyl groups and the lactic acid carboxyl, carbinol, and methyl groups. The radioactivity of the carbon dioxide was measured in a proportional counter, after mixing with methane, as described by Buchanan and Nakao (3).

TABLE I

C^{14}-DISTRIBUTION IN PRODUCTS FROM FERMENTATION OF LABELLED XYLOSE

	C^{14} as % of total in the products				
Product	Xylose-1-C^{14}	Xylose-2-C^{14}	Xylose-5-C^{14}	Wheat xylose	Chemical degradation of wheat xylose*
Acetic acid					
CH_3—	99.7	0.46	0.67	20.0	20.1
—COOH	0.13	99.3	0.04	21.2	20.6
Lactic acid					
—COOH	0.02	0.05	0.67	19.5	20.8
—CHOH—	0.07	0.08	0.13	20.0	20.7
—CH_3	0.07	0.08	98.5	19.3	19.1

* *Data taken from Brown (2).*

Results and Discussion

Acetic and lactic acids were formed from xylose by resting cell suspensions in equimolar amounts as previously reported for the fermentation of arabinose by growing cultures (17). The distribution of C^{14} in these products (Table I) agreed with that expected from scission of the chain between carbon-2 and carbon-3 of the pentose as shown below. The acetic acid from xylose-1-C^{14} was labelled in the methyl group and that from xylose-2-C^{14} in the carboxyl group. Xylose-5-C^{14} gave methyl-labelled lactic acid. There was very little activity in any of the other carbons.

The sample of wheat xylose was the same as that examined by Brown (2) with a chemical method of degradation and his results are reprinted here for comparison. The agreement between the chemical and biological methods is quite good and shows essentially uniform labelling.

The figures in Table I are relative specific activities. The specific activities actually determined (microcuries per millimole) for each of the five carbons were added together and then each was calculated as percentage of this total. This method of calculation corrects for dilution by products formed by fermentation of unlabelled carbohydrate stored in the cells. The specific activities actually measured were only about 85% of what would be expected if there was no dilution.

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The technical assistance of Mr. J. Dyck and Mr. M. D. Chisholm is gratefully acknowledged.

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STUDIES ON THE GLYCOPROTEINS OF THE DOMESTIC FOWL

II. THE HEXOSAMINE CONTENT OF CERTAIN TISSUES OF THE SEXUALLY IMMATURE PULLET AND SOME EFFECTS THEREON OF GONADAL HORMONES[1]

By P. A. Anastassiadis, W. A. Maw, and R. H. Common

Abstract

The concentration of total hexosamine (as free base) in the dry matter of tissues of the pullet was high in cartilage (8.0 mgm. per gm.) and in oviduct (6.4 mgm. per gm.); medium in comb and wattles, tendon, and lungs (3.0 to 5.5 mgm. per gm.); and low in voluntary muscle (1.5 mgm. per gm.). Dry defatted skin contained 8.3 mgm. per gm. Total serum hexosamine was 52 mgm. per 100 ml. and was increased significantly to about 67 mgm. per 100 ml. by treatment with estradiol benzoate (ODB) and to about 72 mgm. per 100 ml. by ODB plus testosterone propionate (TST). Serum protein was also increased significantly by the hormonal treatments, but to a relatively greater extent. The magnum of the hypertrophied oviducts of pullets treated with ODB was relatively high in dry matter (22.0%), and this dry matter was relatively rich in hexosamine (13.8 mgm. per gm.) and low in hydroxyproline (2.9 mgm. per gm.). The dry matter of the uterus and vagina was relatively low in hexosamine (6.0 mgm. per gm.) and rich in hydroxyproline (5.7 and 10.5 mgm. per gm. for uterus and vagina respectively). Concurrent administration of ODB and TST led to a greater degree of hypertrophy of the oviducts than did administration of ODB alone; both the weight and total hexosamine content of the magnum showed a greater relative increase than did the values for vagina and uterus. Some modifications of the method of determining the hexosamine content of tissues are described.

Introduction

During recent years it has become apparent that the two histological entities of the intercellular material, the fibrillar and the interfibrillar, have their chemical counterparts. The principal constituent of the fibrillar material is collagen; and since the ratio hydroxyproline N: total N in collagen is remarkably constant (9), it is possible to measure the collagen content of a tissue from its hydroxyproline content. A major constituent of the interfibrillar material is glycoprotein, this term being used here as meaning protein which on hydrolysis yields a relatively considerable proportion of carbohydrate and, in particular, hexosamine. In contrast to the constancy of the ratio between hydroxyproline and collagen, the relation between hexosamine nitrogen and glycoprotein nitrogen or between hexosamine and interfibrillar substance is variable. Nevertheless, a large proportion of the total tissue glycoprotein is present as hyaluronic acid and chondroitin sulphate, which have a constant hexosamine content. The hexosamine content of tissues should, therefore, provide some measure of the amounts of interfibrillar substances in the tissues and, perhaps, of physiological changes of these amounts.

The present communication forms part of a study of the glycoproteins of the domestic fowl based on the foregoing premise, and falls into the following

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two sections:—(*a*) a survey of the hexosamine content of a number of different tissues, and (*b*) a study of the effects of gonadal hormones on the hexosamine content of certain of these tissues.

Hydroxyproline analyses were also made on many of the samples because the glycoproteins are, in general, rather closely associated with collagen.

Analytical Methods

Determination of Hexosamine

These analyses were made by a method described previously (1), but with the following modifications:—

(*a*) The entire hydrolyzate was not dried over solid NaOH. Instead, aliquots of the hydrolyzate were dispensed immediately after the hydrolysis into 5 ml. volumetric flasks. The flasks were then placed in a vacuum desiccator over solid NaOH, and the desiccator was evacuated to a pressure slightly greater than the vapor pressure of 4 *N* HCl (25 mm. at 25° C.). This procedure shortened the time required in order to dry the hydrolyzate.

(*b*) The aqueous standard hexosamine solutions were replaced by standards made up in 4 *N* HCl. Such standards were found to be stable at room temperature for at least several months. The portions necessary for each set of analyses were pipetted into 5 ml. volumetric flasks and dried in the same way as the aliquots of the hydrolyzate.

(*c*) The amounts of Ehrlich reagent and of ethanolic HCl were increased from 0.5 ml. to 1.0 ml., and the amount of ethanol added was correspondingly reduced from 3.0 ml. to 2.5 ml. This modification facilitated the handling of the carbonate precipitate formed on addition of alcohol and did not affect the mean absolute extinctions.

The magnitudes of the corrections applied for interferences by humin and sugar – amino acids for a number of hydrolyzates (7, 1) were compared with the differences between hexosamine values obtained from analyses of hydrolyzates before and after their passage through a Dowex-50 column (5, 2). The divergences between the two sets of values were small. Although the principle of a corrective blank for sugar – amino acid interaction is applicable for any concentration of acetylacetone–buffer, nevertheless a correction coefficient should be established for each actual concentration of these reagents used by each worker. This coefficient may be calculated from the following relation:—[Extinction at 530 mμ of sugars + amino acids treated with acetylacetone + sodium carbonate]: [Extinction at 530 mμ of sugars + amino acids treated only with sodium carbonate.]

Fig. 1 shows that (*a*) the color given by an invert sugar – lysine mixture when it is heated in sodium carbonate solution is not the same as the color given on heating in acetylacetone – sodium carbonate, and that (*b*) the depth of the latter color is greatly influenced by the acetylacetone concentration. For the concentration of the reagents used in the present work (2.66% acetylacetone in 0.50 *M* Na_2CO_3 in the acetylation medium), the correction coefficient lay close to unity (actual value = 1.07).

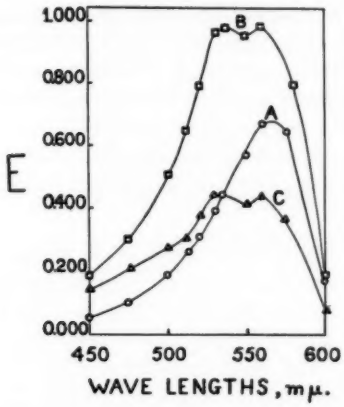

FIG. 1. Extinction curves of a mixture of inverted sucrose – lysine treated with sodium carbonate alone, and sodium carbonate + acetylacetone.
Curve A: mixture heated in 0.5 *M* sodium carbonate only.
Curve B: mixture heated in 0.5 *M* sodium carbonate + 1.59% acetylacetone.
Curve C: mixture heated in 0.5 *M* sodium carbonate + 2.16% acetylacetone.

In the later stages of the work now reported, some samples were also analyzed by a procedure based on hydrolysis with 0.05 *N* HCl in the presence of a large excess of ion exchange resin (20–25 mgm. dried tissue plus 8 ml. 0.05 *N* HCl plus 2 gm. Dowex 50, 200–400 mesh, placed in sealed Pyrex test tubes and heated for 24 hr. at 105° C.–107° C.). After hydrolysis the test tubes were opened and used as an adsorption column. The sugars were removed from the column with water, and the hexosamine was then eluted with 2 *N* HCl. The hexosamine values obtained by this technique were about 90% as great as those obtained by the conventional hydrolysis with 4 *N* HCl followed by the correction procedure given previously (1).

Hydroxyproline Determination

Hydroxyproline was determined by the method of Neuman and Logan (8). The tissue samples were hydrolyzed with 4 *N* hydrochloric acid in sealed tubes heated in boiling water for four hours. The hydrolyzate was either dried under vacuum over sodium hydroxide and redissolved in water (analyses of sera and oviducts) or neutralized with sodium hydroxide (analyses of skin). The factor 7.46 (9) was used for conversion of hydroxyproline to collagen. The analyses for hexosamine and hydroxyproline were carried out on freeze-dried material for all the tissues except serum and skin of the second experiment, for which defatted freeze-dried tissues were used.

Nitrogen

Nitrogen was determined by micro-Kjeldahl.

Serum Lipid and Protein

Two milliliters of serum was extracted twice with 20 ml. ethanol – ethyl ether (3 : 1 by volume). The combined extracts were evaporated on a steam

bath and the residue was dried to constant weight. The lipid was then extracted twice with boiling light petroleum. The residue was dried and reweighed and the difference was taken as a measure of the total lipid. The crude protein was calculated by multiplying the nitrogen content of the fat free serum by 6.25.

Experimental

A. Survey of the Hexosamine Contents of Some Avian Tissues, and the Influence of Estrogen Thereon

Six 12-week-old birds (New Hampshire ♂ × Barred Plymouth Rock ♀), comprising three males and three females, were treated as follows: Two males and two females were heavily estrogenized (6 × 2 mgm. estradiol benzoate) over the last 12 days before killing. The remaining two birds were left untreated as controls. The food consumption of all the birds was kept constant at 80 gm. commercial chick starter per day.

At the end of the experimental period the birds were killed and bled. The skin, pectoralis major, peroneus longus, cartilage (from the knee joint), cnemius tendon, oviduct, lung, combs, and wattles were dissected out, weighed, frozen, and freeze-dried.

The dissected tissues were analyzed for hexosamine and total nitrogen. The results of hexosamine determinations and the results for hexosamine nitrogen expressed as a percentage of total nitrogen of the various tissues are presented in Table I. This percentage affords a more accurate expression of

TABLE I

HEXOSAMINE AND HEXOSAMINE NITROGEN IN DISSECTED AVIAN TISSUES

Tissue	Control birds		Estrogenized birds	
	Male	Female	Male	Female
Hexosamine (base) content (mgm./gm. of dry tissue) and the influence thereon of sex and estrogenic treatment				
Cartilage	8.8	7.2	7.7	7.1
Lung	6.0	4.9	3.9	4.6
Skin	4.4	3.8	3.1	2.3
Oviduct	—	3.3	—	7.7
Tendon	4.1	3.2	2.9	2.1
Comb and wattles	4.0	2.9	2.4	2.9
Pectoralis major	1.6	1.6	1.7	1.4
Peroneus longus	1.5	1.9	1.5	1.7
Hexosamine nitrogen expressed as percentage of total N and effect thereon of sex and estrogen				
Cartilage	0.62	0.49	0.44	—
Lung	0.32	0.27	0.27	0.29
Skin	0.29	0.40	0.28	0.38
Oviduct	—	0.19	—	0.14
Tendon	0.18	0.16	0.14	0.11
Comb and wattles	0.31	0.16	0.15	0.18
Pectoralis major	0.08	0.12	0.09	0.09
Peroneus longus	0.08	0.10	0.08	0.09

the hexosamine content of non-defatted tissues, as it eliminates indirectly the fat of the tissues. The percentage of hexosamine nitrogen in total nitrogen was very high in cartilage (about 0.55); high in skin (0.3), lungs (0.3), oviduct (0.2), combs and wattles (0.25), and tendon (0.15); and low in pectoralis major (0.10) and peroneus longus (0.10).

The data are insufficient to permit of any conclusion on sex differences or on the effects of estrogen on the tissues, except in the oviduct.

B. *The Effects of Estrogen and Androgen on the Fat, Crude Protein, Hexosamine, and Hydroxyproline of Serum*

Serum, oviduct, and skin were selected for more detailed study for the following reasons: (*a*) Many serum constituents are affected by gonadal hormones and it was desirable to ascertain whether hexosamine and hydroxyproline are also affected; (*b*) the growth of the oviduct is under the control of the gonadal hormone activity in the body; and (*c*) the skin may be regarded as a mirror of changes in membranes and epithelial tissues generally.

Fifty-two cross bred pullets (New Hampshire ♂ × Barred Plymouth Rock ♀), aged about eight weeks, were assigned at random to four groups. During the last 10 or 12 days before killing, the groups were treated as follows:—

	Mgm. of daily dosage × days			
	Androgen*		Estrogen**	
Group	First killing	Other killings	First killing	Other killings
1	—	—	—	—
2	1 × 12	1 × 10	—	—
3	—	—	1 × 12	1 × 10
4	1 × 12	1 × 10	1 × 12	1 × 10

* *Testosterone propionate* ("*Oreton*", *Schering*).
** *Estradiol benzoate* ("*Progynon*", *Schering*).

Food consumption was kept constant at 80 gm. commercial chick starter per bird per day. The birds were killed on the morning after the day on which the last injection had been given.

The sera and skin of all the birds, and the vagina, uterus, and magnum of the oviducts of 36 birds, were analyzed for hexosamine and hydroxyproline. Serum and skin were defatted and the total lipid recorded.

The results for serum fat, crude protein, and hexosamine are presented in Table II. The increases of serum lipid and protein evoked by estrogen were in agreement with many previously recorded observations (4, 10). It should be noted, however, that TST brought about a depression of the lipemia evoked

by ODB (comparison of Group 3 with Group 4). A re-examination of comparable results from a previous experiment (3) revealed a similar depression by TST of the lipemia evoked by ODB. It is also worthy of note that, although ODB almost doubled the level of total serum crude protein, concurrent administration of TST did not modify this response.

TABLE II

AVERAGE LIPID, PROTEIN, AND TOTAL HEXOSAMINE CONTENTS OF SERA OF SEXUALLY IMMATURE PULLETS

	Group			
	1	2	3	4
	Treatment and birds in each group			
	Control (12)	TST (12)	ODB (12)	ODB + TST (12)
Serum lipid, gm./100 ml.	0.57 ± 0.07	0.75 ± 0.09	12.4 ± 0.73	10.6 ± 0.58
Serum protein, gm./100 ml.	3.7 ± 0.16	3.4 ± 0.12	6.2 ± 0.14	6.3 ± 0.09
Serum hexosamine, mgm./100 ml.	52 ± 4.7	55 ± 4.0	67 ± 6.6	72 ± 6.0
Ratio: $\frac{\text{hexosamine}}{\text{protein}} \times 100$	1.42	1.60	1.09	1.14

Notes: (*a*) *Figures preceded by* ± *denote standard errors.* (*b*) *For* $P = 0.05$, *the following differences were not significant for any constituent:—Group 1 vs. Group 2, and Group 3 vs. Group 4. All other differences were significant.* (*c*) *For* $P = 0.10$, *differences between Group 1 and Group 2 and between Group 3 and Group 4 for lipid attained significance while those for protein did not.*

The average total serum hexosamine of the control group was 52 mgm./100 ml. This figure carried a high variance (S.E. = ± 4.7). TST alone did not affect the level, a result that might have been expected from the absence of any effect of androgen on total serum protein. ODB increased the average level to 67 mgm./100 ml. and this increase attained significance at $P = 0.05$; concurrent administration of TST did not modify this effect significantly.

The hexosamine nitrogen comprised 0.7 to 0.8% of the total protein nitrogen of the sera of Groups 1 and 2, but only 0.5 to 0.6% of the total protein nitrogen of Groups 3 and 4.

The extra protein fractions that appear in the fowl's serum on treatment with estrogen comprise a considerable proportion of serum phosphoprotein and may have a hexosamine content different from that of the original serum proteins of unestrogenized birds. The following separations of serum were carried out in order to study this point.

Serum samples were obtained from control pullets and comparable birds treated with ODB or ODB + TST. Fifteen milliliters of serum was measured into a 40 ml. centrifuge bottle. The lipoprotein fraction was precipitated by the addition of two volumes distilled water. The bottle was centrifuged and

the supernatant was decanted and reserved. The precipitate was dissolved in a minimum of 10% sodium chloride, and reprecipitated by dilution. The mixture was centrifuged and the supernatant was added to the previous supernatant. The precipitate was suspended in 1% sodium chloride and then reprecipitated by the addition of 20% trichloracetic acid until the formation of precipitate ceased. The precipitate was again separated by centrifugation and was then extracted with ethanol – ethyl ether and then with chloroform–ethanol, dried, weighed, and analyzed for total nitrogen and hexosamine. The proteins of the combined supernatant were precipitated by addition of 20% trichloracetic acid, and treated and analyzed in the same way as was the lipoprotein precipitate. The main analytical results are presented in Table III.

TABLE III

HEXOSAMINE CONTENTS OF THE PRESUMPTIVE PHOSPHOPROTEIN FRACTION OF PULLETS' SERUM AND OF THE RESIDUAL SERUM PROTEINS

	Treatment			
	Control	TST	ODB	TST + ODB
Hexosamine in lipid free phosphoprotein fraction, mgm. per ml. serum	0.05	0.06	0.43	0.55
Hexosamine in lipid-free residual serum proteins, mgm. per ml. serum	0.38	0.38	0.42	0.37
Total hexosamine, mgm. per ml. serum	0.43	0.44	0.86	0.92
Hexosamine in phosphoprotein fraction expressed as per cent	1.85	2.07	1.77	1.77
Hexosamine in residual protein as per cent	1.77	2.00	1.62	1.60

It will be noted that the hexosamine contents of the presumptive phosphoprotein fractions were not essentially different from the hexosamine contents of the rest of the serum proteins, whatever the treatment. The observed differences in hexosamine content of the two fractions as between treatments are not regarded as significant.

The analyses for total hydroxyproline of serum suggested that the amounts present in the sera were minute. It is doubtful, indeed, whether any measurable amount was present. The analyses were hampered by the relatively great irrelevant absorptions. If any hydroxyproline was present in the sera, then the amount did not exceed 0.5 mgm./100 ml. serum in the control group or 1.5 mgm./100 ml. in the estrogenized birds.

C. Hexosamine and Hydroxyproline Contents of Oviduct and the Effects Thereon of Gonadal Hormones

Since the oviducts of Groups 1 and 2 were small and undeveloped, they were analyzed as a whole. The oviducts of the other birds were divided into magnum, uterus (shell gland), and vagina. The isthmus was discarded.

TABLE IV

AVERAGE FRESH AND DRY WEIGHTS OF OVIDUCTS OF SEXUALLY IMMATURE PULLETS AS AFFECTED BY ANDROGEN AND ESTROGEN. SIX BIRDS IN EACH GROUP

Group	Treatment	Part of oviduct	Fresh wt., gm.	Dry wt., gm.	Dry matter %
1	Control	Whole	0.122	0.023	18.7
2	TST	Whole	0.158	0.031	19.4
3	ODB	Magnum	2.58	0.575	22.3
		Uterus	3.40	0.524	15.4
		Vagina	1.24	0.192	15.5
		Sum of 3 parts	7.22	1.291	17.9
4	ODB + TST	Magnum	5.01	1.308	26.1
		Uterus	4.49	0.696	15.5
		Vagina	1.70	0.262	15.4
		Sum of 3 parts	11.20	2.266	20.2

The average weights and the dry matter of the dissected oviduct tissues are presented in Table IV. The oviducts of Group 2 were slightly, but not significantly, heavier than those of Group 1; it is well known (6) that TST in larger doses causes some stimulation of the avian oviduct. The weights of the oviducts of Group 3 showed a response of the expected magnitude. The oviducts of Group 4 were significantly heavier than those of Group 3, this being in accord with the established synergism of androgen and estrogen in regard to hypertrophy of the avian oviduct. It is noteworthy that the additional response evoked by androgen was relatively greatest for the magnum, this being more evident in regard to dry weight than to fresh weight. The difference is accentuated when the dry matters are compared, because under the influence of androgen the hypertrophied magnum of the estrogenized pullet becomes less watery. These observations are summarized in Table V.

The average results for hexosamine and hydroxyproline are presented in Table VI, where the values are expressed both as mgm. per gm. dry weight and as total weight in mgm. The hexosamine content of the magnum was

TABLE V

RELATIVE INCREASES OF MAGNUM, UTERUS, AND VAGINA OF ESTROGENIZED FOWL PRODUCED BY ADDITIONAL ANDROGEN TREATMENT. VALUE WITH ODB ALONE TAKEN AS 100

	Fresh weight	Dry weight
Magnum	194	227
Uterus	132	133
Vagina	137	136
Sum of 3 parts	155	176

the highest of any tissue studied; the values of 13.8 and 18.8 mgm. per gm. dry matter were even higher than the values found for cartilage (*vide* Table I). The uterus contained the lowest concentration of hexosamine, but even here the values were of a higher order than those found for voluntary muscle (*vide* results for pectoralis major and peroneus longus Table I). The concentration of hexosamine in the vagina was slightly higher than in the uterus. The effect of TST in increasing the total magnum hexosamine of estrogenized pullets was greater than its effect on fresh weight and total dry matter, as is shown in Table VII.

Table VI also shows that the dry matter of oviduct (less infundibulum and isthmus) contained 0.52% and 0.39% hydroxyproline in Groups 3 and 4 respectively. The lower figure for Group 4 was due to the relatively greater increase of the magnum in Group 4 as compared with Group 3, the magnum

TABLE VI

HEXOSAMINE AND HYDROXYPROLINE CONTENT OF OVIDUCTS OF IMMATURE PULLETS AS AFFECTED BY ANDROGEN AND ESTROGEN. SIX BIRDS IN EACH GROUP

			Hexosamine		Hydroxyproline	
Group	Treatment	Part of oviduct	Mgm. per gm. dry wt.	Total wt., mgm.	Mgm. per gm. dry wt.	Total wt., mgm.
1	Control	Whole	6.4 ± 1.32	0.15	13.7	0.32
2	TST	Whole	6.6 ± 0.54	0.21	15.9	0.49
3	ODB	Magnum	13.8 ± 0.45	8.0	2.9	1.7
		Uterus	5.5 ± 0.31	2.9	5.7	3.0
		Vagina	6.6 ± 0.40	1.3	10.5	2.0
		Sum of 3 parts	9.4	12.1	5.2	6.7
4	ODB + TST	Magnum	18.8 ± 0.97	24.6	1.8	2.4
		Uterus	6.8 ± 0.32	4.8	5.6	3.9
		Vagina	8.8 ± 0.79	2.2	10.3	2.7
		Sum of 3 parts	13.0	31.5	3.9	9.0

Note: Figures preceded by ± denote standard errors.

TABLE VII

RELATIVE INCREASES OF HEXOSAMINE AND HYDROXYPROLINE OF MAGNUM, UTERUS, AND VAGINA OF ESTROGENIZED FOWL PRODUCED BY ADDITIONAL ANDROGEN TREATMENT. VALUE WITH ODB ALONE TAKEN AS 100

	Total amount with ODB + TST relative to amount with ODB taken as 100	
	Hexosamine	Hydroxyproline
Magnum	308	141
Uterus	166	130
Vagina	169	135
Sum of 3 parts	260	134

being relatively low in hydroxyproline and rich in hexosamine. The concentration of hydroxyproline was considerably higher in the undeveloped oviducts of Groups 1 and 2 but these oviducts, because of their rudimentary character, were not comparable with those of Groups 3 and 4 in anatomical terms. It is clear, however, that the rudimentary oviducts were relatively rich in fibrillar material.

D. Hexosamine and Hydroxyproline Contents of Skin and the Effects Thereon of Treatment with Gonadal Hormones

A portion of skin (0.8 gm. to 2.5 gm.) was stripped from the ventral surface of the body and freed from feathers. No attempt was made to remove such hypodermic fatty tissue as came away on stripping off the skin. The sample was defatted by successive extractions with ethanol – ethyl ether and chloroform–ethanol. The analytical results are presented in Table VIII. Estrogen did not affect the total lipid, but androgen appears to have reduced the lipid whether or not estrogen had been given.

TABLE VIII

AVERAGE HEXOSAMINE AND HYDROXYPROLINE CONTENTS OF SKIN OF SEXUALLY IMMATURE PULLETS AND THE EFFECT THEREON OF TREATMENT WITH GONADAL HORMONES

	Group			
	1	2	3	4
	Treatment and birds in each group			
	Control (12)	TST (8)	ODB (10)	ODB + TST (8)
Fat content of dry skin, %	49.7 ± 2.7	41.1 ± 2.4	53.2 ± 2.9	39.9 ± 3.3
Hexosamine (base), mgm./gm. dry defatted skin	8.0 ± 0.41	8.0 ± 0.43	8.3 ± 0.39	8.4 ± 0.25
Hydroxyproline, mgm./gm. dry defatted skin	43.7 ± 3.7	46.3 ± 4.1	49.7 ± 4.5	45.3 ± 5.6

Note: (*a*) *Figures preceded by ± denote standard errors.*

The total hexosamine concentration was about 8.0% of the dry defatted material, a value as high as the figure for dry cartilage, but lower than that for magnum. There were no significant differences between the groups.

The average hydroxyproline content in the skin of the control birds (43.7 mgm./gm. defatted dry material) corresponds to a collagen content of around 33%. The data provide a suggestion that estrogen increased the collagen content of the skin, but the variance was so great (probably owing to the uncertainties of the skinning and sampling) that none of the differences attained significance.

Discussion

The chemical evidence set forth above suggests that treatment with gonadal hormones brings about striking intercellular changes in the oviduct of immature fowl. The hypertrophy of the oviduct evoked by exogenous estrogen is probably characterized by a relatively greater increase of interfibrillar material than of fibrillar collagen.

The differentiation of the oviduct into its three main parts seems, also, to be characterized by intercellular changes. The formation of magnum as a distinct tissue is probably accompanied by an increase therein of the concentration of interfibrillar glycoproteins and a reduction of the concentration of fibrillar collagen. The formation of the uterus, on the contrary, is not accompanied by any demonstrable change of the glycoprotein content, but it is accompanied by a decrease of collagen. Lastly, the formation of vagina is accompanied with a slight increase of the glycoprotein and a slight decrease of the collagen.

It is necessary to stress that the above changes were realized as a consequence of a high level of dosage with exogenous hormones. It will be of interest, therefore, to study how far the changes involved do, in fact, simulate the changes that occur during the smoother, natural onset of reproductive activity.

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HOMEOSTATIC ADJUSTMENT IN THE RENAL TUBULAR TRANSPORT OF INORGANIC PHOSPHATE IN THE DOG[1]

By James G. Foulks

With the technical assistance of Anne C. Robertson *and* William Martin

Abstract

By means of the infusion of small amounts of sodium sulphate it has been possible to elevate the filtered load of inorganic phosphate to the renal tubule in fasted dogs without the administration of exogenous phosphate. Under these circumstances, the reabsorption of phosphate remains virtually complete, even when filtered loads are reached which result in a substantial phosphaturia when phosphate has been administered. By comparing phosphate reabsorption and excretion in fasted animals, and in animals at various intervals after feeding, the existence of homeostatic adjustments in the renal tubular transport of inorganic phosphate has been demonstrated. The available evidence suggests that the intracellular disposition of phosphate itself may be an important factor in determining the rate of renal tubular phosphate transport at filtered loads in the physiological range. The limitations of the determination of the phosphate "Tm" as a device for studying homeostatic processes have been discussed.

Introduction

According to conventional concepts, the renal excretion of inorganic phosphate is accomplished by glomerular filtration and tubular reabsorption (44). The reabsorptive process is generally considered to possess a definite threshold at which phosphate spills into the urine, and a stable maximal transport capacity. Pitts and his co-workers (2, 30, 39, 41), who have studied phosphate excretion most extensively, have particularly emphasized the stability of the phosphate Tm, its constancy from day to day in a given individual, and its independence of acute shifts in body electrolyte and acid–base balance. While this concept has not passed unchallenged (28), it has gained wide acceptance. A corollary of this view attributes variations in the urinary excretion of phosphate solely to alterations in the filtered load presented to the tubules, i.e. to changes in the rate of glomerular filtration or to extrarenal modifications in the plasma concentration of the ion or its filterability (44). These inferences are not in harmony with the evidence which is accumulating to the effect that a number of renal tubular transport systems have a remarkable degree of flexibility and possess the capacity to vary substantially in response to homeostatic requirements.

Recent investigations of the renal excretion of inorganic phosphate frequently have been based upon the determination of the phosphate Tm (2, 24, 28, 30, 34, 39, 40, 41). Although modifications in the phosphate Tm have been observed under several experimental conditions (2, 24, 28, 34, 40) no attempt has been made to relate these changes to homeostatic requirements. While phosphate excretion is promoted by acidosis (19, 20, 21, 25) and by the

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administration of parathyroid hormone (1, 5, 8, 14, 18, 23, 24), it has not been possible to demonstrate that these circumstances lead to an alteration of the phosphate Tm (28, 30, 39, 41). Numerous observations have indicated that variations in phosphate excretion under these and other circumstances (6, 7, 26, 29, 31, 32, 37, 46) may occur at normal serum phosphate concentrations. However, this phenomenon has not been systematically explored with clearance techniques and the renal mechanisms involved have remained obscure.

The usual diet of most animals contains a fairly large excess of phosphate. While intestinal absorption is often incomplete, a considerable amount of phosphate is absorbed from the bowel and eliminated in the urine. This process extends over a considerable period of time. The bulk of the phosphate disposed of in this manner is excreted at filtered loads insufficient for the saturation of the tubular reabsorptive process (37). A priori, it is entirely reasonable that the threshold at which phosphate spills into the urine might be varied without alteration in the maximal transport capacity. In fact this phenomenon has been noted during acidosis in man (41). The experiments reported in this communication were undertaken in an effort to determine whether or not homeostatic adjustments in the renal tubular transport of inorganic phosphate could be detected at "sub-saturation" filtered loads.

Methods

All experiments were performed on trained, unanesthetized female Golden Labradors, loosely restrained on a comfortable animal board. Creatinine clearances were used for the measurement of the glomerular filtration rate. As an initial priming dose, 4 ml./kgm. of 5% creatinine was administered intravenously, followed by a sustaining infusion of 25–30 mgm./min. Approximately one-half hour was allowed for equilibration before clearance periods were begun. Urine was collected by means of an indwelling wing-tip latex catheter, the bladder being washed with 15 ml. of distilled water at the end of each period. Jugular vein blood was drawn at the mid-point of alternate periods, the samples being taken in oiled syringes and transferred to heparinized tubes for immediate centrifugation. Creatinine was determined by the method of Folin and Wu (9) and inorganic phosphate by the method of Gomori (15). For the determination of filterable phosphate, heparinized plasma was filtered through cellulose dialysis tubing by means of centrifugation. The filtrates contained less than 20 mgm.% protein as determined by the salicylsulphonic acid turbidity.

The dogs were fed a diet consisting of 20 parts of water, 5 parts of codfish frames, 5 parts of beef lungs, 1.5 parts of beef liver, and 20 parts of a balanced ration which contained about 60% carbohydrate and 20% protein, with ample vitamin supplementation. The animals studied after feeding had been given a meal of this standard diet. Its phosphorus content is estimated at approximately two per cent of the total dry weight. The age of the animal may be a factor in its renal handling of phosphate (25). The animals used in these experiments were studied during the period between 17 and 24 months of age.

Results

Phosphate Excretion During Sodium Phosphate Infusion in Fasted Animals

Previous investigations have indicated that as the filtered load of phosphate is increased by phosphate infusion, reabsorption is virtually complete until a threshold is reached when the load approaches a level of about 75% of the Tm. With further increases in load, phosphate excretion increases progressively, and the reabsorptive process becomes saturated when the filtered load exceeds the Tm by a margin of about 50% (39).

Our initial objective was to establish a uniform, fairly constant, filtered load of phosphate at a point intermediate between the threshold and Tm. Accordingly, phosphate was infused at a rather slow rate in the hope that an equilibrium would be reached between the infused and excreted phosphate, stabilizing the filtered load at the desired level. This goal proved difficult to achieve. Increases in glomerular filtration rate as well as in serum phosphate concentration usually were observed. A typical experiment is presented in the first section of Table I. Even at very modest infusion rates (0.33 mgm.* P/min.), the filtered load continued to increase throughout the period of observation, until the rate of phosphate reabsorption approximated the Tm value. The urinary output of phosphate rose to exceed the rate of infusion. The transition zone between threshold and Tm levels was often traversed within two to three clearance periods, a serious limitation to the systematic study of this range of loads.

Phosphate Excretion After Feeding

In the preceding experiments, all animals had been fasted for 18–24 hr. prior to study. This procedure is conventionally adopted in many renal clearance studies in dogs, in order to rule out uncontrolled variables and to provide conditions as nearly uniform as possible from one experiment to another. Our inability to achieve stable intermediate phosphate filtered loads during phosphate infusion led to a series of experiments in animals following oral rather than intravenous administration of phosphate. Since it was hoped that these studies might provide a means for detecting possible alterations in phosphate transport in response to requirements for homeostasis, it was decided that conditions of phosphate loading which occur physiologically (i.e. normal feeding) should be employed. No effort was made to control the amount of food ingested, the dogs being fed "to appetite". However, by performing experiments at various intervals of time after feeding, it was possible to observe a wide range of filtered loads, comparable to that seen with small phosphate infusions in fasted dogs. Data from typical experiments are presented in Tables I*B* and II*A*. The production of fairly stable filtered loads within the desired range was readily accomplished by this technique. The marked and sustained increase in phosphate excretion which characterizes the

* *For purposes of comparison with older studies, phosphate has been expressed as mgm. phosphorus. The reported values can be converted to millimoles as follows: mgm.% ÷ 3.1 = mM./liter, mgm./min. ÷ 31 = mM./min.*

postprandial period is evident. A comparison of the rate of phosphate excretion under the two sets of experimental conditions described above suggests that for a given filtered load, excretion is greater after feeding than with phosphate infusion. In view of the rapidly rising filtered load of phosphate during its infusion, the significance of this difference may be open to question.

TABLE I

RENAL EXCRETION OF PHOSPHATE

Time, min.	C_{Cr}[1]	V[2]	S_P[3]	U_P[4]	L_P[5]	U_PV[6]	R_P[7]
	cc./min.		mgm.% P		mgm./min. P		
A. During the infusion of isotonic, isohydric sodium phosphate in the fasted dog. Dog Louella—wt. 21 kgm.							
−30	Sustaining infusion of six parts of 5% creatinine and one part of isotonic sodium phosphate at 0.7 ml./min.						
0– 20	73	0.8	4.23	2.6	3.1	0.02	3.1
20– 40	71	0.6		1.9		0.01	
40– 60	72	0.7	4.85	1.8	3.5	0.01	3.5
60– 80	72	1.2		2.6		0.03	
80–100	77	1.2	5.43	6.7	4.2	0.08	4.1
100–120	85	1.5		14		0.21	
120–140	90	1.9	5.75	19	5.2	0.35	4.8
140–160	92	1.8		27		0.47	
160–180	93	1.2	5.96	35	5.4	0.43	5.0
180–200	97	1.0		51		0.49	
200–220	99	0.9	5.98	76	5.9	0.66	5.2
220–233	250 ml. isotonic sodium phosphate containing 390 mgm. creatinine I.V.						
233	Sustaining infusion of isotonic sodium phosphate containing 1% creatinine at 3 ml./min.						
260–280	105	3.5	15.5	313	16.3	10.8	5.6
280–302	114	3.3	14.1	325	16.0	10.8	5.3
B. After feeding. Dog Louella—wt. 17.7 kgm.							
−4 hr.	Fed						
−30	Sustaining infusion of two parts of 5% creatinine and one part of Na_2SO_4 (150 milliosmols per liter) at 0.75 ml./min.						
0– 20	102	0.8	3.64	75	3.7	0.62	3.1
40– 60	99	1.7	3.56	66	3.5	0.46	3.1
60– 80	115	1.0		66		0.67	
80–100	95	0.7	3.58	79	3.4	0.52	2.9
100–120	99	0.8		66		0.50	
120–146	98	0.7	3.83	82	3.8	0.59	3.2
146–166	100	0.8		62		0.48	
166–186	92	0.6	4.01	90	3.7	0.49	3.2
186–206	95	0.7		95		0.62	
206–226	94	0.6	4.04	112	3.8	0.72	3.1
226–239	250 cc. isotonic sodium phosphate containing 390 mgm. creatinine I.V.						
239	Sustaining infusion of isotonic sodium phosphate containing 1% creatinine at 3 ml./min.						
266–289	95	2.0	14.4	500	13.8	9.8	4.0
289–309	105	1.9	13.6	526	14.2	10.2	4.0

[1] C_{Cr} — *Glomerular filtration rate.*
[2] V — *Rate of urine flow.*
[3] S_P — *Serum phosphate concentration.*
[4] U_P — *Urine phosphate concentration.*
[5] L_P — *Filtered phosphate load.*
[6] U_PV — *Rate of urinary phosphate excretion.*
[7] R_P — *Rate of phosphate reabsorption.*

TABLE II

RENAL EXCRETION OF PHOSPHATE

Time, min.	C_{Cr}[1] cc./min.	V	S_P	U_P mgm.% P	L_P	U_PV mgm./min. P	R_P
A. After feeding. Dog Louella—wt. 21.8 kgm.							
−10 hr., 40 min.	Fed						
−30 min.	Sustaining infusion of three parts of 5% creatinine and two parts of Na_2SO_4 (150 milliosmols per liter) at 1.0 ml./min.						
0– 20	110	1.0	4.39	150	4.8	1.54	3.3
20– 40	106	0.8		150		1.24	
40– 60	105	0.8	4.64	142	4.9	1.15	3.7
60– 80	109	0.7		160		1.16	
80–100	115	0.8	4.90	156	5.6	1.25	4.4
100–120	102	0.8		153		1.14	
120–140	101	0.7	5.00	162	5.1	1.15	3.9
140–160	102	0.7		153		1.12	
160–180	99	0.8	4.90	149	4.9	1.17	3.7
180–200	105	0.7		150		1.13	
200–220	102	0.7	4.51	148	4.6	1.03	3.6
220–233	250 ml. isotonic sodium phosphate containing 390 mgm. creatinine I.V.						
233	Sustaining infusion of isotonic sodium phosphate containing 1% creatinine at 3 ml./min.						
260–280	117	2.4	14.3	442	16.6	10.9	5.8
280–300	123	3.0	13.4	360	16.4	10.8	5.7
B. During the infusion of sodium sulphate in fasted dog. Dog Louella—wt. 20.5 kgm.							
−30	Sustaining infusion of two parts of 5% creatinine and one part of Na_2SO_4 (150 milliosmols per liter) at 0.75 ml./min.						
0– 20	78	0.7	3.36	1.0	2.6	0.01	2.6
20– 40	77	0.7		1.0		0.01	
40– 60	75	0.9	4.10	1.0	3.1	0.01	3.1
60– 80	81	0.9		1.0		0.01	
80–100	80	0.9	4.39	1.0	3.5	0.01	3.5
100–120	94	1.0		1.0		0.01	
120–140	97	0.9	5.11	1.0	5.0	0.01	5.0
140–160	101	0.8		1.0		0.01	
160–180	108	0.8	5.08	1.1	5.5	0.01	5.5
180–200	112	0.8		2.0		0.02	
200–220	112	0.8	5.33	5.7	6.0	0.04	5.9
220–233	250 ml. isotonic sodium phosphate containing 300 mgm. creatinine I.V.						
233	Sustaining infusion of isotonic sodium phosphate containing 0.83% creatinine at 3 ml./min.						
260–280	121	2.5	14.3	442	17.3	11.0	6.3
280–307	138	2.4	12.8	485	17.6	11.4	6.3

Phosphate Excretion During Sodium Sulphate Infusion in Fasted Animals

A considerable elevation of the glomerular filtration rate was a common occurrence during the course of our experiments. Increases of 30–50% above the initial values were typically observed. These made a substantial contribution to the increase in the filtered phosphate load. This circumstance suggested the possibility that the desired range of filtered loads might be accomplished by this factor alone, in the absence of phosphate administration. However, when 5% creatinine alone was infused, the increases in filtered load were not adequate for a comparison with the experiments in which phosphate had been administered.

In all of the preceding experiments on fed animals, small amounts of sodium sulphate were added to the infusion in order to promote an adequate urine flow and to insure the accurate estimation of the rate of phosphate excretion. In the fasted animal, this technique was found to produce consistent elevations in the serum phosphate concentration, often to values as high as those found after feeding, in addition to the usual increase in filtration rate. In the terminal periods of experiments of this type, the filtered phosphate loads approximated the maximum levels observed after feeding or after slow phosphate infusion. We were thus provided fortuitously with a technique for comparing phosphate excretion in fasted animals who received no exogenous phosphate and in fed animals, at an identical range of filtered loads. The administration of creatinine and sodium sulphate at comparable rates of infusion in both series of experiments provided conditions which were uniform except for the interval after feeding. The protocol of a typical experiment is depicted in Table II*B*. It is apparent that at filtered loads which produce a consistent and significant degree of phosphate excretion following phosphate administration, tubular reabsorption is virtually complete and the urine remains essentially phosphate-free when the filtered load is elevated without the administration of phosphate. This finding has been repeated consistently in animals which have been fasted for 18–24 hr. preceding experimentation.

The marked difference in the rate of phosphate excretion at comparable filtered loads in fed and fasted dogs is illustrated by the mass data presented in Fig. 1. In 102 clearance periods from 10 experiments in fasted animals, the rate of phosphate excretion did not exceed 0.25 mgm./min., and in 90% of the observations was less than 0.10 mgm./min. In 64 clearance periods from

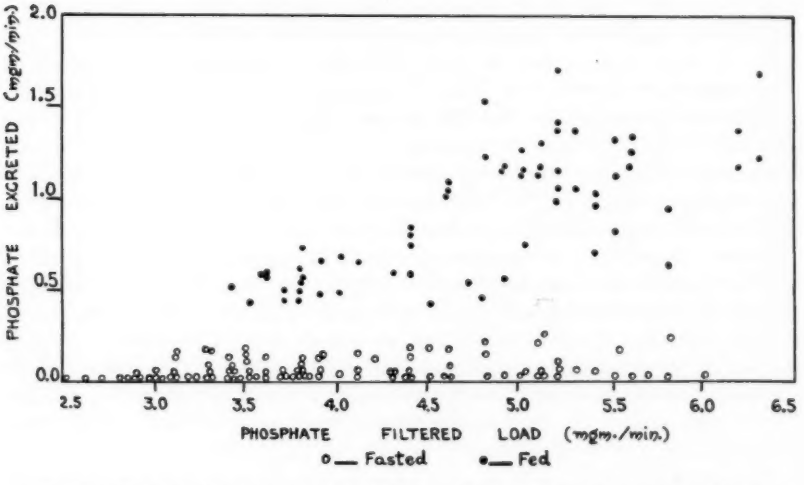

FIG. 1. Rate of phosphate excretion in fed and fasted dogs over a comparable range of filtered loads.

eight experiments which were begun between 3 and 11 hr. after feeding, the phosphate excretion rate was never less than 0.40 mgm./min., and exceeded 1.0 mgm./min. in three-fourths of the periods in which the filtered load was greater than 4.5 mgm./min.

The difference in the rate of phosphate excretion between fed and fasted animals is not dependent on differences in filtration rate, serum phosphate concentration, or urine flow. Table III presents data from selected periods taken from two representative pairs of experiments, one after fasting and the other after feeding, for each of the two dogs studied. The differences in phosphate excretion and reabsorption are a uniform finding at essentially identical values for the other measured variables of renal function.

TABLE III

COMPARISONS OF PHOSPHATE EXCRETION IN FED AND FASTED DOGS AT ESSENTIALLY IDENTICAL URINE FLOWS, FILTRATION RATES, AND SERUM PHOSPHATE CONCENTRATIONS

Type of experiment	V	C_{Cr}	S_P	U_P	L_P	U_PV	R_P
	cc./min.		mgm.% P		mgm./min. P		
Dog: Baby							
Fasted	0.4	99	3.5	1	3.5	0.01	3.5
Fed	0.5	96	3.7	101	3.6	0.60	3.0
Dog: Louella							
Fasted	0.8	101	5.1	1	5.1	0.01	5.1
Fed	0.7	101	5.0	162	5.1	1.15	3.9

For practical purposes, it is often assumed that plasma inorganic phosphate is completely filterable through the glomerular membrane. There is a substantial body of evidence that the bulk of the plasma inorganic phosphate will pass through cellulose membranes into protein-free filtrates or dialyzates (44). A small and variable fraction of the plasma phosphate is usually found to be non-diffusable in this type of experiment. It seemed important to determine whether or not the differences in the rate of phosphate excretion observed under the present experimental conditions were associated with appreciable differences in plasma phosphate filterability. Filtrates of plasma were obtained by means of centrifugation through cellulose dialysis tubing. The phosphate concentration in the filtrates ranged from 83 to 98% of that found in the samples of whole plasma from which they were derived. Illustrative data are presented in Table IV. The data show that when the estimated filtered load has been elevated in the fasted dog by means of slow sodium sulphate infusion, the negligible excretion of phosphate cannot be attributed to decreased phosphate filterability. Nor is the phosphaturia observed after feeding associated with an increase in phosphate filterability. The comparability of the actual filtered load of phosphate under these circumstances was thus confirmed.

A number of investigators have observed a discrepancy in the simultaneous specific activity of urine and plasma following the injection of radioactive

TABLE IV

FILTERABILITY OF PLASMA INORGANIC PHOSPHATE

Dog	Experimental condition	U_PV, mgm./min.	L_P, mgm.P/min.	S_P*, mgm.% P	Filtered S_P, mgm.%	% Filterable†
Louella	Fasted—control period	0.03	3.28	3.22	2.67	83
	Fasted—after 250 min. of slow Na_2SO_4 infusion	0.01	5.72	5.42	5.06	93
Baby	Fasted—control period	0.01	3.45	3.48	3.24	93
	Fasted—after 290 min. of slow Na_2SO_4 infusion	0.04	5.32	4.04	3.73	92
Louella	Five hours after feeding	0.39	5.50	4.31	3.90	90

** The serum phosphate values have not been corrected for the protein content of the plasma (approximately 5 gm.% in these animals) and are therefore about 5% less than the actual concentration of phosphate in plasma water.*

† Correction for plasma water concentration would reduce these figures by 4–5%. The actual freedom of phosphate to diffuse may have been even less than the measured values would indicate, since complete filterability would be expected to produce a filtrate concentration of phosphate in excess of 100%, if distribution is in accordance with the Gibbs–Donnan equilibrium.

phosphate and have concluded that "exogenous" inorganic phosphate is excreted more readily than "endogenous" phosphate (12, 13, 16, 17, 38). Other workers have disputed this finding or have made another interpretation of their data (22, 27, 47). Inasmuch as the differences reported are rather transitory (less than one hour in duration), they do not offer an adequate explanation for the increased rate of phosphate excretion observed a number of hours after phosphate administration. The phosphaturia which occurs at "sub-saturation" filtered loads following phosphate administration could conceivably take place without modification in tubular transport if a fraction of the inorganic phosphate were complexed in some fashion which prevented its reabsorption without altering its filterability. Substantial evidence in support of such an hypothesis is lacking.

It is clear that the fasted animal is capable of reabsorbing phosphate at filtered loads well in excess of those usually present, if the load is raised without the administration of phosphate. It seems reasonable to conclude that the increased excretion of phosphate at filtered loads of 4 to 6 mgm./min. which occurs following the administration of phosphate (whether orally or intravenously) is the result of a modification of the tubular transport of phosphate under these circumstances. While the elevation of the filtered load contributes to the increase in phosphate excretion, increases in load of this magnitude are not sufficient to produce appreciable phosphaturia in the absence of altered tubular function. The observed alteration of tubular transport facilitates the excretion of the excess phosphate and is therefore in a direction which would assist in the achievement of phosphate homeostasis.

Phosphate Tm Values in Fed and Fasted Animals

At the conclusion of a number of experiments, a phosphate load was infused and the phosphate Tm determined. The values for three determinations

under each set of experimental conditions from a single animal were as follows: after fasting; 3.5, 4.2, and 5.0 mgm./min. (average = 4.2); after feeding: 3.8, 4.3, and 5.3 mgm./min. (average = 4.5). This finding is in accord with that of Ayer, Schiess, and Pitts (2) who found no difference in the phosphate Tm of dogs three hours following the ingestion of a meat meal as compared with fasted animals. It is apparent that the determination of the phosphate Tm fails to provide a means for demonstrating the significant difference in the renal transport of this ion under these two sets of experimental conditions.

Discussion

Phosphate Reabsorption in the Fasting State

The ability of the fasted animal to excrete a urine which is practically free of phosphate is well known. We have shown that this ability extends to filtered loads considerably in excess of those usually encountered in the fasting state. Apparently the demonstration of this phenomenon requires that the filtered load be elevated without the administration of exogenous phosphate. The infusion of sodium sulphate was found to produce this particular set of circumstances.

Undoubtedly, the effects of sodium sulphate infusion are not confined to an elevation of the serum concentration and filtered load of inorganic phosphate. While the rate of sodium sulphate infusion in these experiments is uniformly small (15–25 μM./min.), nevertheless the possibility that this experimental condition promotes phosphate reabsorption is worthy of consideration. Osmotic diuresis per se does not have such an effect when large amounts of phosphate are infused. The diuresis produced by urea (28) and mannitol (34) has been reported to be without influence on the phosphate Tm. The infusion of large amounts of sodium sulphate has been reported to produce a urine of very low chloride content (43), the presence of an abundance of unreabsorbed anion presumably permitting chloride reabsorption to proceed more nearly to completion. Neither of these mechanisms is adequate to explain the differences in phosphate reabsorption reported here, since comparable amounts of sodium sulphate have been infused in both fed and fasted animals. We have also observed that the infusion of sulphate in these amounts has no effect on the pattern of phosphate excretion during the infusion of phosphate at slow rates. If sulphate infusion promotes phosphate reabsorption in the fasted animals, it fails to do so when phosphate is administered. It appears unlikely that the rate of phosphate reabsorption is altered by sulphate infusion itself. However, this assumption is not required for the conclusion that the tubular response to an elevated filtered load of phosphate is altered following phosphate administration.

Phosphate Reabsorption Following Phosphate Administration

The data presented above show that when the filtered load of phosphate is increased by the administration of phosphate either orally or intravenously, the rate of phosphate excretion is substantially greater than when the filtered

load is elevated without the administration of exogenous phosphate. The question arises whether the depression of phosphate reabsorption seen after feeding can be attributed solely to the ingestion of phosphate. While the infusion of amino acids has been shown to depress the phosphate Tm, the serum level of these substances which is attained after a meat meal is not of the order of magnitude required to produce this effect (2). The possibility that a postprandial increase in plasma glucose concentration may depress phosphate reabsorption should be considered, in view of the report that phlorizin enhances the phosphate Tm, a finding which has been interpreted as indicating that phosphate reabsorption is depressed by glucose at normal plasma glucose levels (39). While the possible role of dietary constituents other than phosphate cannot at present be evaluated, the fact that phosphate infusion alone also leads to increased phosphate excretion at the range of filtered loads involved in the current experiments would appear to offer an adequate explanation for the changes seen after feeding.

Previous investigations have provided evidence that intravenous phosphate administration can alter renal tubular transport of phosphate. Several studies have called attention to the fact that during prolonged phosphate infusion in the cat (7), dog (28), and human (34), the phosphate Tm tends to diminish with time, sometimes to as little as one-fourth of its initial value. Eggleton and Habib (7) found that following the infusion of substantial quantities of phosphate in the anesthetized cat, phosphate reabsorption is considerably reduced as compared with control values, when the plasma concentration and filtered load of phosphate are allowed to return to normal levels. We have made the same observation in the anesthetized dog.

The present experiments do not elucidate the mechanism whereby the adjustment in phosphate reabsorption following phosphate administration is mediated, although one or two possible explanations may be excluded. The phenomenon of "fatigue" or of "self-depression", in the sense usually employed (i.e. autoinhibition of transport enzyme at high substrate concentration) (42), cannot be involved in the adjustments under consideration, since the filtered loads were comparable in both types of experiments and were for the most part less than those required to saturate the tubular reabsorptive mechanism.

The serum phosphate concentration is not the determining factor, since comparable levels were produced in each type of experiment. McCrory (33) has studied the renal response to an oral phosphate load in children, by comparing normal children with those subjected to moderate phosphate depletion by means of aluminum hydroxide ingestion. At comparable serum phosphate levels and filtered loads, the renal reabsorption of phosphate was uniformly greater in the phosphate depleted subjects. This finding is in accord with the hypothesis that the cellular content of phosphate is the important determinant of the rate of phosphate reabsorption. This interpretation parallels that previously advanced with respect to the stimulus leading to an augmented potassium secretion by the renal tubule (11, 36). Hogben and Bollman (28) have provided evidence that changes in serum

potassium may be related to the changes in phosphate Tm seen with prolonged phosphate infusion. In view of the prominent position which these two components occupy in the ionic pattern of intracellular fluid, it would not be surprising if their mechanism of homeostatic adjustment should prove to be interrelated and perhaps to share a number of features in common. In this connection, it is of interest that Taugner *et al.* (45) have recently reported that tubular secretion of inorganic phosphate can be demonstrated in the cat when phosphate esters are infused. While convincing evidence of the existence of a secretory mechanism for phosphate has not been obtained in other species, this possibility cannot be excluded. Barclay, Cooke, and Kenney (3) have favored the hypothesis that phosphate is both reabsorbed and secreted by the canine renal tubule. Two other ions, potassium (4, 35) and thiosulphate (10), are now known to be handled in this fashion. In the present discussion of phosphate transport, the term reabsorption carries no implication other than the net resultant of tubular transport activity.

The Measurement of Maximal Transport Capacity

The determination of the phosphate Tm has become a generally accepted procedure for evaluating the status of the renal tubular phosphate transport process. The infusion of a large phosphate load is a prerequisite for this measurement. Indeed, it has been emphasized that the filtered load must exceed the Tm by a margin of about 100% if the values for phosphate reabsorption are to be significant (39). The results reported here indicate that this procedure cannot in fact measure the actual capacity of the tubules to transport phosphate, since the experimental conditions required modify the process being studied. It can only measure the maximal transport capacity under that particular set of experimental conditions, i.e. under heavy intravenous phosphate loading. The depression of phosphate reabsorption which follows phosphate infusion will obviously lead to an underestimation of the maximal capacity for transport. Prolonged infusion is not required for this effect to be manifest. In about one-third of the experiments in which we have measured the phosphate "Tm", we have found it to be considerably less than the rate of phosphate reabsorption observed in earlier periods of the same experiment, prior to the infusion of phosphate. The actual differences in phosphate reabsorption observed in these experiments are presented in Table V.

The concept that the renal tubular transport of phosphate is rather inflexible can be attributed to the fact that its response to the administration of a large intravenous load is rather uniform under a variety of experimental conditions. The similarity of the phosphate Tm in fed and fasted animals demonstrates that the technique required for measuring the "Tm" is capable of obscuring important homeostatic adjustments, and may well mask the effect of other influences which are capable of modifying this transport process, including factors of major physiological importance. This would seem to be a possible explanation for the difficulty which has been encountered in attempts to demonstrate modifications in the phosphate "Tm" under conditions which are

TABLE V

MAXIMAL PHOSPHATE REABSORPTION OBSERVED PRIOR TO AND FOLLOWING PHOSPHATE INFUSION IN SELECTED EXPERIMENTS

Dog	Experimental condition	Maximal reabsorption before I.V. loading, mgm./min.	Phosphate Tm* after I.V. loading, mgm./min.	Difference
Baby	Fasted	3.9	3.5	0.4
Baby	Fasted	5.6	5.0	0.6
Baby	Fed	5.1	4.3	0.8
Louella	Fed	4.8	3.7	1.1
Louella	Fed	5.9	4.5	1.4

** All Tm values represent the average of two successive periods following the elevation of the filtered phosphate load to 12–15 mgm./min. by the infusion of large amounts of isotonic phosphate.*

known to produce physiological alterations in phosphate excretion. The limitations of the usefulness of the Tm concept which are suggested by these experiments may not be confined to phosphate transport. It seems probable that previous investigations, in which increases in endogenous phosphate excretion in fasted animals have been attributed to increases in filtration rate or serum phosphate concentration, actually represent the effect of alteration in renal tubular phosphate transport. The techniques described here should furnish the opportunity to test this possibility.

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A NOTE ON THE STOICHIOMETRY OF ADSORPTION OF ANIONS BY LYSOZYME[1]

By J. Ross Colvin

Abstract

Ten adsorbed anions of monovalent methyl orange, Orange II, or picric acid are necessary and sufficient to precipitate one lysozyme molecule at pH 5.5 in 0.05 *M* acetate buffer at 32°, whereas only five of divalent 2,4-dinitro-1-naphthol-7-sulphonic acid (flavianic acid) are required. These results are consistent with a hypothesis of interacting hydration effects.

Introduction

When a protein or related polymer adsorbs ions in aqueous solution, the adsorption isotherm is usually an approximation to a Langmuir hyperbola which may be interpreted by assuming Coulomb attraction between independent charged centers on the polymer and the ion (6, 7, 9). Recently, however, anomalous sigmoid isotherms have been observed for systems in which anions are precipitated by positively charged polymers and these have been attributed to either a solubility product relation (10) or to interacting hydration effects (1, 3, 4). In one such system, each lysozyme molecule has been shown to be associated with 10 methyl orange anions over at least the lower three fourths of the total adsorption capacity (4). This observation is consistent with both possibilities mentioned above but additional data may show which is correct. Examination of similar systems was therefore begun but had to be suspended. The initial results are recorded below. The anions of Orange II, 2,4-dinitro-1-naphthol-7-sulphonic acid (flavianic acid), and of picric acid are shown to be adsorbed stoichiometrically also by positively charged lysozyme and the implications of this fact are considered.

Materials and Methods

Samples of lysozyme and the sodium salts of the anions were the same as previously used (1, 3, 4). Experimental techniques and methods of analysis were as described before (4). The negligible effect of lysozyme in the solution upon the extinction coefficient of the anions was confirmed.

Results

A redetermination of the relation between the number of moles of methyl orange adsorbed by lysozyme and the weight of lysozyme precipitated is shown in Fig. 1. As found previously (4), adsorption of 10 methyl orange anions is necessary and sufficient to precipitate each lysozyme molecule in 0.05 *M* acetate, pH 5.5, at 32°. Fig. 1 also shows that an equal number of Orange II and picrate anions is required under the same conditions, although

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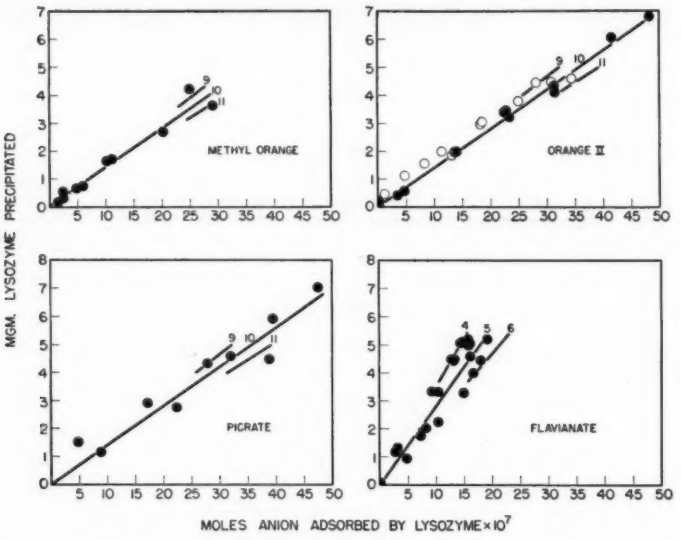

FIG. 1. The relations between amount of lysozyme precipitated and moles of various anions adsorbed in 0.05 *M* acetate, pH 5.5, 32° C. (The open circles for Orange II indicate points obtained at 3° C.). The number of anions adsorbed per lysozyme molecule which is consistent with the indicated slope is given at the end of each line.

the molar free energy of adsorption of these two ions differs widely (1). The greater scatter of points for the picrate ion is due to its lower adsorption. In contrast, only five anions of flavianate are required to precipitate one lysozyme molecule in the same buffer. Here too, scatter of the points is wide because of low intensity of adsorption of the ion and the small number of anions bound per protein molecule.

An attempt was made, where practicable, to determine the effect of temperature upon the ratio of the components in the complex. Fig. 1 gives the results for Orange II-lysozyme at 3° C. and 30° C. in 0.05 *M* acetate, pH 5.5. Clearly, for this system, the effect is negligible. Similar information for the other complexes could not be obtained because of the low intensity of adsorption of the anion or its low solubility at 3° C.

Discussion

These observations confirm and extend conclusions stated previously (4). In addition, because flavianate is divalent under the conditions used while the other anions are monovalent, the results suggest that neutralization of 10 positively charged groups per molecule is required to precipitate the lysozyme. If 10 divalent anions are assumed simultaneously to neutralize nearly independent charges on the surfaces of adjacent lysozyme molecules, as required by a solubility product relation, one should expect to find evidence

for intermediate complexes. Such evidence was not detected (4). Furthermore, if the electrostatic effects of such groups on a single lysozyme molecule were nearly independent, at least ten of the charged sites would have to be grouped so that a divalent anion could neutralize two simultaneously. An approximate calculation of the maximum area occupied by five flavianate molecules (8) shows that it is less than 10% of the area of the lysozyme surface (2). Therefore unless one half of the accessible positively charged groups on a molecule of lysozyme are so arranged on only 10% of the surface, the electrostatic effects of the groups on the protein cannot be independent. Although no detailed information is available on the distribution of groups upon the lysozyme surface (5), the latter possibility seems more probable. This conclusion is consistent with the hypothesis of interacting hydration effects previously suggested for these systems (4) but leaves unanswered the problem of why the effective neutralization of just ten charges is sufficient.

Acknowledgments

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RELATIONSHIP OF OXYGEN CONSUMPTION TO BODY TEMPERATURE IN THE RESTRAINED RAT[1]

By Roscoe G. Bartlett, Jr.[2], Vernon C. Bohr, and William I. Inman

Abstract

Forty adult Sprague-Dawley rats were divided into four groups: 10 control and 10 restrained animals at room temperature (23° C. ± ½°) and 10 control and 10 restrained animals at 0° C. ± ½°. Continuous recordings were made on oxygen consumption and body temperature. It was learned that the restrained animals had an initially higher oxygen consumption than the control animals. This gradient was maintained throughout a three-hour exposure in the case of the animals maintained at room temperature but in the case of the animals maintained in the cold it was reversed early in the tests, i.e., the oxygen consumption of the restrained animals fell below that of the control animals. The fall in oxygen consumption was accompanied by a fall in body temperature. From the data it was not possible to state which was the cause and which was the effect. It was suggested that both decreased oxygen consumption and temperature drop may be the effect of another cause, emotionality or emotional stress.

Several investigators (2–6) have demonstrated that light restraint renders the smaller laboratory animals incapable of maintaining body temperature in a cold environment. It was felt that a study of the relationship between body temperature fall and metabolism might indicate a cause–effect relationship between these two parameters.

Methods and Materials

Forty adult male Sprague-Dawley rats (250–260 gm.) were divided into four groups: 10 control animals at room temperature, 10 restrained animals at room temperature, 10 control animals at 0° C., and 10 restrained animals at 0° C. The metabolism chamber was constructed from a 1 gal. widemouthed jar which was then laid on its side. The carbon dioxide produced was absorbed by a layer of barium lime spread beneath the wire grid on which the animals were maintained. The oxygen consumption was continuously recorded with a small, precision spirometer fitted with an ink writer and a recording kymograph. The accuracy of the method was such that the introduction of 5 cc. of air into the system produced a deflection as calculated from the dimensions of the spirometer bell. For any test the error was less than five per cent. The metabolism chamber was immersed in a water bath of the same temperature as the air (room temperature 23° + ½° and cold room temperature 0° C. + ½°). Because animals of nearly identical weights were used the oxygen consumptions are reported in cc. per animal per hour.

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Contribution from the Department of Physiology, College of Medical Evangelists, Loma Linda, California and Department of Physiology, School of Medicine, Howard University, Washington 1, D.C.

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[2] *Present address: Department of Physiology, Howard University, Washington 1, D.C.*

Body temperatures were recorded intermittently with small indwelling thermocouples (inserted in the rectum to a depth of 7.5 cm. to read high colonic temperature) in the restrained animals. In order that the movement of the animals would not dislodge the thermocouple from the rectum during the tests this depth of insertion was used. This depth of insertion is not injurious to the animals as there has been no case of rupture in many hundreds of tests. The thermocouples were smooth and non-traumatizing. They were periodically calibrated against the laboratory thermometers and readings of both instruments on the same animal checked within $\frac{1}{2}$° C., which was sufficiently accurate for the type of measurement made. Because of the freedom of movement allowed to the control animals, only initial and terminal temperatures were taken with laboratory thermometers inserted to a depth of 7 to 8 cm. for two minutes or longer—until there was no change in the reading. The control animals were maintained free in the chamber while the restrained animals were placed in a restraining cage which was then placed in the metabolism chamber. The restraining cage had a circumference of 9 in. and a length of 9 in. Many animals were able to effect an end for end reversal in the cage. A finger or pencil could easily be inserted between the animal's back or sides and the cage. Thus, while the animal was confined to a very restricted area, body movements were possible and, as mentioned below, the animals were seen to struggle, many of them continuously, so that the movement of the restrained animals was more than that of the control animals. Since the animals were placed in the restraining cages just prior to the beginning of the tests very little time elapsed before the beginning of the measurements.

Results

The body temperature and oxygen consumption of the restrained and control rats at room temperature are shown in Fig. 1. Similar data for the rats maintained in the cold are shown in Fig. 2. The volumes of oxygen consumption are corrected to standard conditions. The ordinates in both figures have been scaled so that a 50% drop in metabolism corresponds to a 10° C. drop in body temperature, i.e., if the relationship between body temperature drop and metabolism corresponded to a Q_{10} of 2 the curves would be parallel.

Several observations can be made from an analysis of the curves: (1) maintenance in the cold resulted in an initially higher metabolic rate of both restrained and control animals than rats kept at room temperature; (2) restraint and the resultant struggling resulted in an increased metabolism as compared to control animals under the same conditions (only initially in the restrained animals in the cold); (3) initial metabolism of the restrained animals in the cold was higher than the control animals but soon fell below that of the control animals; and (4) restrained animals at room temperature maintained, throughout the test, a metabolism higher than that of the control animals.

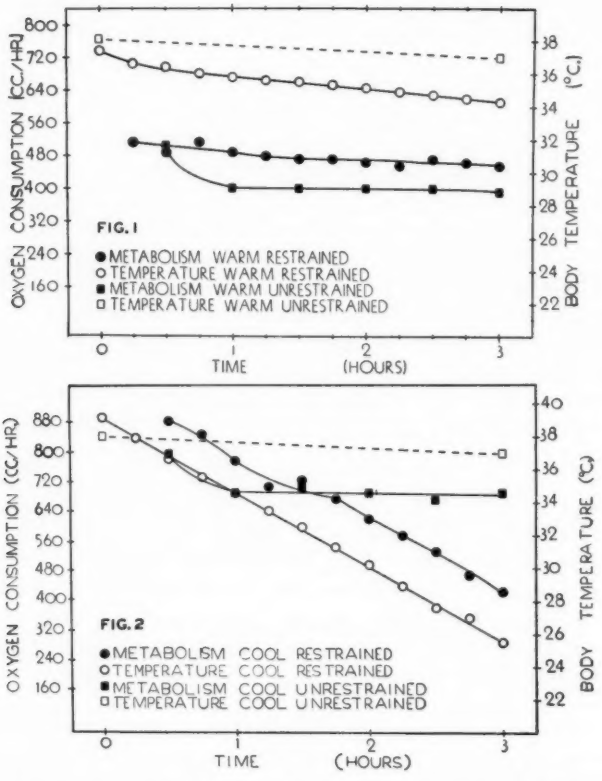

FIG. 1. Oxygen consumption and body temperature of restrained rats maintained at room temperature (23° C. ± ½°).

FIG. 2. Oxygen consumption and body temperature of restrained rats maintained at cold room temperature (0° C. ± ½°).

Discussion

If one assumes that restriction of breathing, as maintained by some (7), was essential for the production of restraint (emotional) hypothermia, then it should follow that the metabolic rate, as measured by oxygen consumption, should be reduced. In these studies however, we obtained an oxygen consumption rate in the restrained animals in the cold which was initially greater than that for the control animals, and at room temperature an oxygen consumption rate for restrained animals which was continuously greater than that recorded for control animals maintained under the same conditions save for the restraint. It appears, therefore, that if there was a restriction of breathing it was not sufficiently marked to prohibit the restrained animals from establishing a metabolic rate higher than that for the non-restrained control animals.

It is also evident from a comparison of the temperature – oxygen consumption curves for the control and restrained animals maintained in the cold room that the restrained animals were capable of making the initial overtures necessary for maintaining a normal body temperature. In fact, as mentioned above, the initial metabolic rates of the restrained animals were higher than those for the control rats. This initial response probably reflects the greater activity of the restrained animals (2, 3). The subsequent fall in body temperature must be explained either on the basis of an increased heat dissipation or a decreased metabolism, or both.

If one assumes a Q_{10} of 2, which is approximately the Q_{10} of mammalian enzyme systems, it is not possible, from these data, to determine the cause and effect relationship between change in oxygen consumption and change in body temperature. The curves run so nearly parallel that it is impossible to ascertain the possibility of the existence of such a relationship. If these two parameters (oxygen consumption and body temperature) are related as cause and effect or effect and cause, the lag between the one and the other is brief and indeterminate by our methods. Of course the possibility exists that both are the effect of another variable. Since we have been able to demonstrate a positive correlation between emotionality (as measured by the "open field test" (Hall, J. Comp. Psychol. 18 : 385. 1934)) and temperature stability (1), this may well be the third variable.

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THE BIOSYNTHESIS OF CELL WALL CARBOHYDRATES

II. FORMATION OF CELLULOSE AND XYLAN FROM LABELED MONOSACCHARIDES IN WHEAT PLANTS[1]

By A. C. Neish

Abstract

D-Glucose-1-C^{14}, D-allose-1-C^{14}, D-ribose-1-C^{14}, D-xylose-1-C^{14}, and sedoheptulose-2-C^{14} were administered to Thatcher wheat plants. The cellulose and xylan were isolated after a 5-48 hr. period of metabolism, and converted to glucose and xylose, respectively. The distribution of C^{14} in both glucose and xylose was then determined by fermentation with *Leuconostoc mesenteroides*. Glucose was found to be a better precursor of both cellulose and xylan than any of the other sugars. The distribution of C^{14} in the products strongly suggested that the main route for synthesis of the xylose units of xylan was by removal of carbon-6 from a hexose and that pentoses were converted to xylan only through a hexose intermediate.

Introduction

In the first paper of this series (7) it was found that D-glucose-1-C^{14} administered to wheat plants was incorporated into cellulose to a considerable extent without rearrangement of the carbon skeleton of the glucose molecule. However an appreciable fraction of the glucose molecules had undergone rearrangement. The present paper is an extension of this work in which certain other sugars are compared with glucose as precursors of cellulose. The biosynthesis of xylan, the other major cell wall polysaccharide of cereals, has also been investigated. It has been found that glucose is superior to the other sugars examined as a precursor of both cellulose and xylan.

Experimental

Materials

D-Glucose-1-C^{14} was prepared by the method of Isbell *et al.* (14). The methods used for preparing D-xylose-1-C^{14} (15), D-ribose-1-C^{14}, and sedoheptulose-2-C^{14} have also been described elsewhere (9, 15, 16). D-Allose-1-C^{14} was made by reduction of D-allonic lactone-1-C^{14}. This lactone was obtained as a by-product in the synthesis of sedoheptulose-2-C^{14} (16). The reduction was carried out in an oxalate buffered medium (14) using the same conditions as were used in the preparation of xylose (15). Crystalline D-allose-1-C^{14} was isolated in a yield of 75%, based on the lactone or 31.5% based on the labeled cyanide. The preparation of allose-1-C^{14} is easily carried out by the cyanhydrin synthesis because of the readiness with which both the lactone and sugar crystallize. Although D-allose is not known to be a naturally occurring sugar, our interest in it was aroused by the observation (3) that it was readily fermented by *Aerobacter aerogenes*.

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The samples of sugars administered to the plants had the following specific activities (μc./mM. of sugar)—ribose (4.37), xylose (6.90), allose (73.1), glucose (10.4), and sedoheptulose (91.0).

Activation of Plants

Thatcher wheat plants were grown in soil in a greenhouse during March and April, with some supplementary light to give 16 hr. of illumination each day. The temperature was kept between 22° to 27° C. The radioactive sugars were administered to these plants by two different methods.

In one set of experiments tillers were cut from young plants (27 days from seeding). Healthy tillers each bearing four leaves were selected and the cut ends immersed in 0.5 ml. of an aqueous solution of the labeled sugar in a 12 x 100 mm. test tube. Two tillers were put in each tube and three tubes (six tillers) were used for each treatment. The tillers were then placed between opposing fluorescent light tubes in a fume hood and were illuminated constantly from opposite directions, with a light intensity of 650-700 foot-candles at 21°-22° C. Most of the radioactive solution was absorbed in one hour due to transpiration. When the tube was nearly dry, distilled water (0.2-0.3 ml.) was added and when this had been almost entirely absorbed the tillers were removed from the tubes and the bases rinsed with water. They were immediately put in a 125 ml. Erlenmeyer containing 30-40 ml. of distilled water and again illuminated for the remainder of the period allowed for metabolism of the absorbed material. Leakage of radioactive material back into the water was negligible and absorption of the labeled compound was quantitative.

The second set of experiments was run with older plants which were flowering (47 days from seeding). These plants were at a stage when lignin synthesis is known to be rapid (18). They were placed in a dark chamber for 24 hr. and then a solution of the radioactive sugar (2-10%) was administered by injection, usually into the second internode, as described previously (7). About 0.2 ml. was injected into each tiller and two or three tillers were used for each treatment. The plants were then placed in a greenhouse and left for two diurnal cycles (48 hr. starting April 21).

These two treatments differed in the age of the plants and the longer time allowed for metabolism in the older plants with alternate dark and light periods. In addition the older plants were still attached to the root system while only the shoots of the young plants were used. For sake of convenience these treatments will be compared by referring to "young" and "old" plants throughout this paper but it must be kept in mind that the age may not be the variable responsible for any differences obtained.

Isolation and Degradation of Cellulose and Xylan

When the period allowed for metabolism of the radioactive compound was finished the tillers were at once cut into sections about 2-3 cm. in length and dried overnight in a vacuum oven previously heated to 60°-70° C. The dried

samples were ground to pass the 60-mesh screen of a micro Wiley mill and then stored in tightly stoppered glass vials.

Holocellulose was prepared from 2.0 gm. samples of this dried and ground material. Each sample was put in a sintered glass crucible and suspended under a reflux condenser in a widemouth flask, where it was leached with rapidly refluxing methanol for three hours. This treatment extracted the pigments and other components which totalled about 25% of the dry weight. The residue in the crucible was dried and then given three successive chlorite extractions (1). Each extraction was made with 1.5 gm. of sodium chlorite and 0.12 ml. of glacial acetic acid in 100 ml. of water at 75°-80° C. for 45 min. The insoluble residue was centrifuged out between each treatment and the supernatant liquid decanted. After the final extraction this residue (holocellulose) was washed, twice with water and once with methanol, in a centrifuge tube, and finally with methanol and then with ether in a sintered glass crucible under suction. The holocellulose obtained was nearly white. It composed 45-50% of the total dry weight, with no appreciable differences in the yield from young and old plants.

The holocellulose (1.0 gm.) was extracted with 10 ml. of 10% potassium hydroxide for two hours at room temperature (22°-24° C.) The residual cellulose was filtered out, washed with 5 ml. of 10% potassium hydroxide, with 10 ml. of water, and then with 20 ml. of 20% acetic acid. The combined filtrate and washings were treated with three volumes of absolute ethanol to precipitate the xylan fraction. The crude xylan was filtered out and washed with methanol and ether. The cellulose was also washed, finally, with these solvents. Both samples were dried at room temperature in a desiccator. The holocellulose from the young plants gave 45% of cellulose and 32% of xylan while that from the older plants yielded 48% cellulose and 41% xylan.

The cellulose (0.4 gm.) was converted to glucose by hydrolysis with 20 parts of 42% hydrochloric acid (22). The hydrolysis was carried out for 24 hr. at room temperature (22°-25° C.) in a glass-stoppered Erlenmeyer flask. The dark hydrolyzate was poured into 10 parts of water, filtered, and then passed through a column containing 100 ml. of Amberlite IR-4B resin. The neutral effluent was concentrated under reduced pressure to about 5 ml. This fraction was shown by paper chromatography to contain cellobiose and higher oligosaccharides in addition to glucose. A monosaccharide fraction was isolated by chromatography on a Celite 535-Darco G-60 column (21). A column 20 × 185 mm. was used and 200 ml. of water passed through it during two to three hours. The first 60 ml. of effluent was discarded, and the next 125 ml. saved. This second fraction contained practically all the glucose. No other sugars could be demonstrated in it by paper chromatography using butanol/pyridine/water (6/4/3) and developing the spots with a sensitive alkaline silver technique (20). The glucose recovered in this fraction was about 65% based on the cellulose as measured by hypoiodite titration. Some carbonization occurred during hydrolysis and better yields would probably have been obtained with less concentrated hydrocholoric acid or a lower

temperature (22). The glucose crystallized readily on evaporation of the purified solution but, since no other sugars were present, it was not considered necessary to isolate crystalline glucose. The clear, colorless solution was sterilized and then fermented with *Leuconostoc mesenteroides* to determine the distribution of C^{14} in the glucose molecule (2).

The xylan (0.3-0.4 gm.) was refluxed one and one-quarter hours with 40 parts of 3% nitric acid (12). The hydrolyzate was cooled, filtered from a small amount of insoluble gum after addition of Celite analytical filter aid, passed through a column containing 25 ml. of Amberlite IR-4B resin, and the neutral effluent evaporated under reduced pressure to about 5 ml. This was then purified by chromatography on charcoal as described above. The monosaccharide fraction consisted mainly of xylose but small amounts of glucose and arabinose were present so it was necessary to isolate crystalline xylose. The mixture was evaporated to dryness after addition of 1 millimole of pure unlabeled xylose. This residue crystallized. It was dissolved in 0.2 ml. of hot water then 0.3 ml. of methanol and 0.6–0.8 ml. of absolute ethanol was added. The mixture was refrigerated; xylose crystallized out overnight. The crystalline xylose was filtered out, washed with ethanol, then with ether, and air-dried. This xylose was degraded by fermentation with *Leuconostoc mesenteroides* (2) to determine the distribution of C^{14} in the molecule.

The fermentative degradation of these sugars has been found to be a relatively rapid and convenient method. The figures for the percentage distribution are based on the sum of the separate determinations. This is done to correct for the dilution (10–15%) caused by unlabeled carbohydrate in the bacterial cells, and is discussed elsewhere (2). The C^{14} determinations were made by counting $C^{14}O_2$ gas according to the procedure of Buchanan and Nakao (8). A sensitive and accurate method was needed because of the low specific activity of some of the sugars that were available for this work. The lowest counting rates that are reported to two significant figures were about 300 counts per minute above the background.

Results

The treatments given are outlined in Table I. The effectiveness of the various sugars as precursors of cellulose and xylan can be compared by referring to the dilution figures given in the last two columns. Those sugars undergoing the least dilution are the most efficient precursors. It will be noted that allose was the least effective, and glucose the most effective of the sugars as a precursor of either cellulose or xylan. It is noteworthy that glucose was superior to xylose or ribose as a source of carbon for xylan synthesis. Sedoheptulose was also utilized for polysaccharide synthesis. It appears that the rates of synthesis of cellulose and xylan were about equal in the "young" plants but in the "older" plants the rate of xylan synthesis was three to four times as rapid as the rate of cellulose synthesis, no matter which sugar was fed.

TABLE I

INCORPORATION OF C^{14} INTO CELLULOSE AND XYLAN BY WHEAT PLANTS METABOLIZING LABELED MONOSACCHARIDES

Expt. No.	Sugar fed	Dose per gm. of dry plant material		Age of plant, days	Duration of treatment, hr.[1]	% of C^{14} fed found in holocellulose[2]	mμc./mM. of carbon		Dilution of isotope[3]	
		μM. sugar	mμc. of C^{14}				Cellulose	Xylan	Cellulose	Xylan
1	D-Ribose-1-C^{14}	14.2	620	27	5	6.9	2.17	2.43	403	360
2	D-Xylose-1-C^{14}	9.1	630	27	5	6.2	1.61	2.06	860	670
3	D-Allose-1-C^{14}	1.55	1140	27	5	1.4	0.28	0.61	43,500	20,000
4	D-Glucose-1-C^{14}	8.4	870	27	5	16.1	8.80	8.50	198	202
5	Sedoheptulose-2-C^{14}	2.14	1950	27	5	4.3	8.47	11.3	1530	1150
6	Sedoheptulose-2-C^{14}	1.81	1650	27	24	14.2	13.80	20.0	940	650
7	D-Ribose-1-C^{14}	11.1	490	47	48	6.0	0.76	2.85	1150	306
8	D-Xylose-1-C^{14}	9.9	690	47	48	6.8	1.23	5.15	1120	268
9	D-Allose-1-C^{14}	1.61	1190	47	48	1.2	0.26	1.45	47,000	8400
10	D-Glucose-1-C^{14}	9.7	1010	47	48	8.5	2.72	9.32	640	187

[1] *Experiments 1–6 were carried out with constant illumination; Experiments 7–10 represent two diurnal cycles, as described in text.*
[2] *Most of the C^{14} was found in the methanol extract.*
[3] *Specific activity of carbon dioxide obtained on combustion of original sugar divided by specific activity of carbon dioxide obtained on combustion of the polysaccharide.*

TABLE II

DISTRIBUTION OF C^{14} IN GLUCOSE AND XYLOSE ISOLATED FROM THE CELLULOSE OR XYLAN OF PLANTS ACTIVATED WITH D-GLUCOSE-1-C^{14}

	C^{14} as % of total in the monosaccharide molecule			
	Young plants (Expt. 4, Table I)		Old plants (Expt. 10, Table I)	
Carbon atom No.	Glucose	Xylose	Glucose	Xylose
1	81.9	88.0	67.5	60.5
2	2.2	2.1	5.1	5.7
3	2.0	3.2	4.0	5.2
4	1.4	2.0	2.3	2.8
5	0.2	4.7	1.9	25.7
6	12.3	—	19.2	—

About 80% of the glucose that was converted to cellulose was incorporated without rearrangement of the carbon skeleton (see Table II) in the shoots of young plants. In the older plants, which were also given a longer treatment including dark periods, more rearrangement occurred but most of the glucose was still incorporated without prior fission and resynthesis. The xylose isolated from all plants activated with glucose-1-C^{14} was labeled mainly in carbon-1 although that from the older plants had quite a large amount of C^{14} in carbon-5. The glucose isolated from the cellulose of plants activated with ribose-1-C^{14} showed considerable concentration of C^{14} in certain carbons although all carbons had an appreciable concentration of the isotope (see Table III). It is interesting that the xylose from the same plants should have the C^{14} spread around as much as it is in the glucose. Even when xylose-1-C^{14} was administered the xylose reisolated from the xylan showed a widespread redistribution of the isotopic carbon.

TABLE III

DISTRIBUTION OF C^{14} IN GLUCOSE AND XYLOSE ISOLATED FROM THE CELLULOSE OR XYLAN OF PLANTS ACTIVATED WITH LABELED PENTOSES

	C^{14} as % of total in the monosaccharide molecule			
	Activated with D-ribose-1-C^{14}			Activitated with D-xylose-1-C^{14}
	Expt. 1, Table I		Expt. 7, Table I	Expt. 8, Table I
Carbon atom No.	Glucose	Xylose	Xylose	Xylose
1	38.0	49.5	41.4	36.3
2	10.9	12.6	19.0	19.0
3	15.5	14.1	14.2	12.1
4	8.4	8.3	6.3	9.5
5	4.7	15.5	19.1	23.1
6	22.5	—	—	—

Sedoheptulose-2-C^{14} gave rise to glucose and xylose each labeled mainly in carbons 2 and 5 (see Table IV). The xylose carbon skeleton could have been formed from the glucose skeleton by removal of carbon-6. This is particularly evident with the short treatments. When 24 hr. was allowed there was relatively more isotope found in xylose carbon-1, suggesting that xylan is more readily reabsorbed from the cell wall and recycled than is cellulose. This is also suggested by the data in Table II where the xylose formed from glucose in the old plants (longer treatment) was more heavily labeled in carbon-5 than that from the young plants (short treatment). The data in Tables II and III also support the hypothesis that xylose is formed by elimination of carbon-6 of glucose. In addition there appears to be another route whereby glucose-1-C^{14} gives rise to xylose-5-C^{14}.

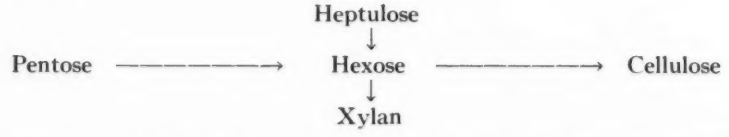
TABLE IV

DISTRIBUTION OF C^{14} IN GLUCOSE AND XYLOSE ISOLATED FROM THE CELLULOSE OR XYLAN OF WHEAT PLANTS ACTIVATED WITH SEDOHEPTULOSE-2-C^{14}

	C^{14} as % of total in the monosaccharide molecule			
	5 hr. metabolism (Expt. 5, Table I)		24 hr. metabolism (Expt. 6, Table I)	
Carbon atom No.	Glucose	Xylose	Glucose	Xylose
1	5.1	8.6	6.9	15.1
2	40.0	43.2	36.9	37.2
3	9.4	7.9	9.7	9.7
4	5.4	6.2	6.9	8.3
5	32.8	34.1	32.5	29.7
6	7.3	—	7.1	—

Discussion

It appears from the foregoing results that both cellulose and xylan are formed from glucose or a related hexose derivative. The synthesis of the two major cell wall polysaccharides in wheat plants can be represented by the scheme:



This is supported by the following facts (*a*) glucose is more rapidly converted to xylan than is xylose or ribose, (*b*) pentoses are converted to xylan only after as much rearrangement of the carbon skeleton as occurs in their conversion to cellulose, and (*c*) glucose fed directly, or formed in the plant from sedoheptulose, appears to be converted to xylan units with loss of carbon-6.

These results are in agreement with an old theory of pentosan formation, i.e.

$$\text{Hexosan} \xrightarrow{\text{oxidation}} \text{Hexuronide} \xrightarrow{\text{decarboxylation}} \text{Pentosan}.$$

This "decarboxylation" theory was suggested to early workers by the structural resemblances between the hexosans and pentosans found in the same plant material. It has fallen into disfavor (5, 6, 11, 13, 17) because modern work has shown that the pentosans often have units with a different ring size or a different point of attachment than the hexosans from the same source. This makes it seem unlikely that the changes postulated can occur with polysaccharides although in the present instance it is possible since the conversion of cellulose to xylan involves no change in linkage or ring size.

The modern view is that pentoses are formed and then condensed to pentosans. It has been suggested that formation of a pentose phosphate occurs by combination of dihydroxyacetone phosphate and glycolaldehyde under the influence of aldolase (5, 11). Pentoses can also be formed from glucose by elimination of carbon-1 through formation of 6-phosphogluconate followed by decarboxylation to ribose-5-phosphate. The enzymes necessary for this have been found in spinach (4). Although either or both of these systems may be essential for formation of pentose phosphates in wheat plants it is apparent from the data tabulated above that they are not important, in a quantitative sense, for formation of a precursor of xylan.

The major route for xylan synthesis might, however, involve glucuronic acid. Decarboxylation of this acid could, hypothetically, give rise to an "active pentose" which is then converted to xylan. The "active pentose" can be visualized as a xylose-coenzyme complex which is not formed readily from a free pentose or a pentose phosphate, but is formed in conjunction with decarboxylation of the uronic acid. This theory will explain such results as have been obtained and is free from the objections raised against the old "decarboxylation" theory (13).

The conversion of pentose to hexose has been studied previously by Gibbs and Horecker (10) using cell free preparations from spinach leaves. Their results could be explained to a large extent by the combined action of the enzymes transaldolase and transketolase. This would result in pentose-1-C^{14} giving hexose with two-thirds of the isotopic carbon in carbon-1 and the remainder in carbon-3. Sedoheptulose-7-phosphate is an intermediate in this transformation. Even with cell free preparations appreciable amounts (8–9%) of the C^{14} were found in carbon-4 and -6. The results obtained above in living plants (Table III) show a more widespread distribution of the isotopic carbon. Some of the hexose could have been formed from the pentose by the action of transaldolase and transketolase but other reactions are occurring which give glucose with carbon-3 and -6 coming from carbon-1 of pentose.

Recent work on the metabolism of uniformly labeled sedoheptulose in plants (19) has shown that it is converted readily to sucrose and that transaldolase is probably active in this transformation. The experiments with

sedoheptulose-2-C^{14} (Table IV) suggest that this was the major route of sedohelptulose metabolism in wheat plants. Transfer of the first three carbons from sedoheptulose to triose phosphate by transaldolase coupled with breakdown and resynthesis of the hexose by aldolase would be expected to give glucose labeled in carbon-2 and -5 with somewhat more activity in carbon-2. This was actually found to be true. The action of aldolase can also explain the labeling in carbon-6 of glucose isolated from plants activated with glucose-1-C^{14} (Table II).

The general distribution of C^{14} in small amounts in all carbons of the sugars isolated might be due to formation of radioactive carbon dioxide followed by reassimilation since the conditions used were favorable for photosynthesis. It is perhaps surprising that the isotopic carbon was not more uniformly distributed considering the relatively long duration of the experiments and the presence of both oxygen and light.

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THE EFFECTS OF THIOURACIL AND OF THYROXINE ON CERTAIN BIOCHEMICAL RESPONSES OF THE IMMATURE PULLET TO COMBINED TREATMENT WITH ESTROGEN AND ANDROGEN[1]

By R. H. Common[2], W. A. Maw[3], and D. S. Layne[4]

Abstract

Sexually immature pullets were given a combined estrogen–androgen treatment, consisting of estradiol benzoate (ODB) plus testosterone propionate (TST). The influences of thiouracil and of thyroxine on various responses to this treatment were studied in two experiments. Thiouracil depressed slightly the hypertrophy of the oviduct evoked by ODB + TST; thyroxine enhanced the hypertrophy in one experiment, but depressed it in the other. Thiouracil enhanced, and thyroxine depressed the hypertrophy of the liver evoked by ODB + TST. Thiouracil also enhanced the increase of liver crude protein per kgm. live weight evoked by ODB + TST. The slight increase of liver deoxyribonucleic acid phosphorus (DNAP) per kgm. live weight evoked by ODB + TST was also enhanced by thiouracil and depressed by thyroxine. The great increase of liver pentose nucleic acid phosphorus (RNAP) per kgm. live weight evoked by ODB + TST was enhanced by thiouracil, but was depressed by thyroxine. The great increase in the ratio RNAP : DNAP in liver evoked by ODB + TST was not modified by thiouracil, but it was depressed by thyroxine. In one experiment, ODB + TST increased kidney weight per kgm. live weight; and this effect was enhanced by thiouracil and by thyroxine. In the other experiment, ODB + TST did not affect kidney weight, but the kidney weight per kgm. live weight was greater in the groups receiving either thiouracil or thyroxine than in the control group. ODB + TST did not affect kidney DNAP or RNAP per kgm. live weight, and a slight increase in kidney RNAP : DNAP observed in both experiments fell short of significance at $P = 0.05$. Both kidney RNAP and DNAP were significantly greater in the groups receiving thiouracil or thyroxine than in the control groups. ODB + TST evoked a slight increase in the ratio RNAP : DNAP in kidney, and this effect was greatly enhanced by either thiouracil or thyroxine. In confirmation of previous work, the hypercalcemia evoked by ODB + TST was depressed by either thiouracil or by thyroxine.

Introduction

It was found in previous work (8) that thiouracil depressed the increases of serum calcium, serum riboflavin, and serum vitamin A evoked by treating the sexually immature pullet with estrogen. In these respects the effects of thiouracil were similar to those of thyroxine (6). However, thiouracil was found at the same time to increase the hypertrophy of liver and oviduct

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[2] *Professor of Agricultural Chemistry.*

[3] *Professor of Poultry Husbandry.*

[4] *National Research Council Bursar 1953–54.*

TABLE I

EFFECTS OF THIOURACIL AND OF THYROXINE IN MODIFYING THE RESPONSE OF THE SEXUALLY IMMATURE PULLET TO TREATMENT WITH ESTROGEN

Organ or tissue	Effect of estrogen	Modification of effect of estrogen by	
		Thiouracil	Thyroxine
Oviduct weight	+ Large	Enhanced (8)	No change (6)
Serum Ca level	+ Large	Depressed (8)	Depressed (6)
Serum riboflavin	+ Large	Depressed (8)	Depressed (6)
Serum vitamin A	+ Large	Depressed (8)	Depressed (12)
Serum phosphoprotein	+ Large	—	Depressed (6)
Liver weight	+ Large	Enhanced (8)	Depressed (6)
Liver crude protein per kgm. live weight	+ Large	Enhanced (8)	Depressed (6)

induced by estrogen; and in these respects the effects were in contrast to those of thyroxine. For convenience of subsequent reference the main results of these previous experiments are summarized in Table I.

The present paper describes two further experiments on the influences of thiouracil and of thyroxine on the responses of the immature pullet to exogenous gonadal hormones. The gonadal hormonal treatment selected for study was a combined treatment with estradiol benzoate (ODB) and testosterone propionate (TST) designed to simulate the endogenous gonadal hormone activity of the puberal pullet. The ultimate aim of this work is a fuller understanding of normal puberal changes conditioned by endogenous gonadal hormones. These changes are more nearly simulated by a concurrent treatment with estrogen and androgen than by estrogen alone. For this reason, it was decided to concentrate attention on the influences of thiouracil and of thyroxine on responses to such a combined treatment. It would obviously have been desirable to include a study of the influence of thiouracil and thyroxine on responses to estrogen alone, but it was not feasible to work on the necessary large scale.

The experiments now described were largely concerned with comparative effects on the liver and kidney and on the pentose nucleic acid phosphorus (RNAP) and deoxyribonucleic acid phosphorus (DNAP) contents of these organs.

Experimental

1. *Method of Experiment*

(*a*) *Experiment 1*

Twenty-four crossbred (New Hampshire ♂ × Barred Rock ♀) immature pullets were assigned at random to four groups each of six pullets. The birds were 63 days old at the beginning and 75 days old at the end of the experi-

ment. The birds were fed on a commercial poultry starter. Food intake was restricted to 80 gm. per day per bird. The birds were housed in individual cages situated at random on the laboratory benches, and were placed in these cages one week before the start of the experiment.

The treatments are shown in Table II. The birds in group A received injections of 0.5 ml. of sesame oil only, while the birds in groups B, C, and D received the hormone dosages by intramuscular injection in a similar volume of sesame oil. Thiouracil was incorporated in the diet for group C at a level of 0.75%. The birds in group D were given 1.0 mgm. per day of thyroxine sodium (British Drug Houses Ltd.) (0.5 ml. of an aqueous solution at pH 7) by intravenous injection.

The birds in groups A, B, and C remained in excellent condition throughout the experiment. Those in group D became increasingly agitated and overactive. They consumed their food allowance very rapidly after each feeding, and they did not gain weight very rapidly. One bird in group D died on the ninth day of the experiment.

On the 13th day of the experiment the birds were weighed and then killed by decapitation and bleeding.

(*b*) *Experiment 2*

Experiment 2 was a complete replication of experiment 1, except that:— (i) treatments were given for 10 days instead of 12 days, and (ii) the thyroxine was administered subcutaneously instead of intravenously.

Each group originally contained seven birds, but one bird in group D died on the seventh day and one bird in group A died by accidental strangulation. One bird was, therefore, discarded at random from each of the groups B and C. The birds were 82 days old on the 11th day of the experiment, when they were weighed and killed by decapitation and bleeding.

2. General Analytical Methods

The oviduct, spleen, liver, kidneys, heart, and thyroids of each bird were removed and weighed.

The preparation of liver and kidneys for analysis, and the determinations of RNAP and DNAP, as well as the determination of serum calcium, crude protein, and dry matter, were carried out as described elsewhere (13).

3. Results and Discussion

The numerical results of both experiments are presented in Tables II-V, and the qualitative findings are summarized in Table VI. The results in so far as they concern groups D apply to survivors, for it is almost certain that the deaths in these groups were a consequence of the high dosage of thyroxine. The live weight gains of the birds receiving thyroxine were lower than those of the controls in both experiments, and this effect attained statistical significance in experiment 2. The following effects of the treatments on the weights of organs were observed:—

TABLE II

EFFECTS OF THIOURACIL AND OF THYROXINE ON RESPONSES OF THE IMMATURE PULLET TO COMBINED TREATMENT WITH ESTROGEN AND ANDROGEN.
EXPERIMENT 1. AVERAGE RESULTS

	Group A	Group B	Group C	Group D	LSD* (a)	LSD* (b)
	No. of birds					
	6	6	6	5		
ODB, mgm.	Nil	12 × 1.0	12 × 1.0	12 × 1.0	—	—
TST, mgm.	Nil	12 × 0.25	12 × 0.25	12 × 0.25	—	—
Thyroxine sodium, mgm.	Nil	Nil	Nil	12 × 1.0	—	—
Thiouracil in feed, %	Nil	Nil	0.75	Nil	—	—
Initial live wt., kgm.	0.85	0.90	0.86	0.87	NS**	
Final live wt., kgm.	0.94	0.98	0.96	0.94	NS	
Increase in live wt., kgm.	0.09	0.08	0.10	0.07	0.03	0.04
Oviduct wt., gm.	0.13	15.1	13.9	17.1	1.48	1.52
Spleen wt., gm.	1.9	1.4	1.4	1.8	0.52	0.69
Heart wt., gm.	4.5	4.7	4.9	7.8	0.44	0.64
Thyroid wt., mgm.	64	72	91	41	13.3	12.8
Liver wt., gm.	20.2	32.1	29.4	25.5	2.7	3.5
Liver wt., gm./kgm. live wt.	21.4	33.1	41.3	26.9	3.4	3.9
Liver crude protein, gm./kgm. live wt.	4.03	5.58	6.95	5.42	0.61	0.47
Liver dry matter, %	28.1	27.7	27.6	25.0	1.4	1.5
Kidney wt., gm.	6.9	7.1	8.8	10.2	1.04	1.05
Kidney wt., gm./kgm. live wt.	7.3	7.3	9.2	10.9	0.95	1.13
Kidney crude protein, gm./kgm. live wt.	1.40	1.48	1.63	2.08	0.15	0.15
Kidney dry matter, %	21.3	22.5	20.7	22.8	1.7	1.8
Serum calcium, mgm./100 ml.	11.3	65.8	33.9	28.7	6.2	6.8

** LSD denotes least significant difference (P = 0.05) by the "F" test (11) between (a) Groups A, B, C or (b) between Group D and any other group.*
*** NS denotes non-significance by "F" test of any group difference.*

TABLE III

EFFECTS OF THIOURACIL AND OF THYROXINE ON THE RESPONSE OF NUCLEIC ACIDS OF LIVER AND KIDNEY TO COMBINED TREATMENT WITH FSTROGEN AND ANDROGEN.
EXPERIMENT 1. AVERAGE RESULTS

	Group A	Group B	Group C	Group D	LSD* (a)	LSD* (b)
	Treatment					
	Nil	ODB + TST	ODB + TST Thiouracil	ODB + TST Thyroxine		
Liver						
DNAP mgm./100 gm. liver	31.4	22.8	20.4	28.1	3.7	3.9
DNAP mgm./kgm. live wt.	6.72	7.60	8.36	7.51	1.34	1.39
RNAP mgm./100 gm. liver	81.6	108.9	100.0	99.6	16.9	14.5
RNAP mgm./kgm. live wt.	17.4	36.1	41.0	27.2	5.8	5.7
Ratio RNAP/DNAP	2.58	4.81	4.92	3.43	0.46	0.44
Kidney						
DNAP mgm./100 gm. kidney	47.9	46.1	41.2	43.3	5.9	6.3
DNAP mgm./kgm. live wt.	3.49	3.37	3.78	4.70	0.61	0.69
RNAP mgm./100 gm. kidney	36.8	36.4	38.2	35.5	6.7	6.3
RNAP mgm./kgm. live wt.	2.72	2.65	3.51	3.85	0.73	0.70
Ratio RNAP/DNAP	0.77	0.81	0.93	0.83	0.15	0.18

** LSD denotes least significant difference (P = 0.05) by the "F" test (11) between (a) Groups A, B, C or (b) between Group D and any other group.*

TABLE IV

EFFECTS OF THIOURACIL AND OF THYROXINE ON RESPONSE OF THE IMMATURE PULLET TO COMBINED TREATMENT WITH ESTROGEN AND ANDROGEN. EXPERIMENT 2. AVERAGE RESULTS

	Group A	Group B	Group C	Group D	LSD* (a)	LSD* (b) a
	No. of birds					
	6	6	6	6		
ODB, mgm.	Nil	10 × 1.0	10 × 1.0	10 × 1.0	—	—
TST, mgm	Nil	10 × 0.25	10 × 0.25	10 × 0.25	—	—
Thyroxine sodium, mgm.	Nil	Nil	Nil	10 × 1.0	—	—
Thiouracil in feed, %	Nil	Nil	0.75	Nil	—	—
Initial live wt., kgm.	0.84	0.86	0.85	0.81	NS**	
Final live wt., kgm.	1.19	1.24	1.20	1.08	NS	
Increase in live wt., kgm.	0.35	0.38	0.35	0.27	0.07	0.07
Oviduct wt., gm.	0.20	16.3	12.8	12.6	2.2	2.1
Spleen wt., gm.	2.5	2.1	1.7	1.9	0.52	0.57
Heart wt., gm.	5.1	5.1	5.0	8.2	0.36	0.39
Thyroid wt., mgm.	77	83	106	54	23.1	11.3
Liver wt., gm.	25.3	41.9	39.3	27.4	5.3	5.5
Liver wt., gm./kgm. live wt.	22.8	33.9	41.2	26.6	4.2	3.6
Liver crude protein, gm./kgm. live wt.	4.52	5.47	7.77	5.18	0.62	0.50
Liver dry matter, %	28.7	28.2	27.4	25.6	1.4	1.2
Kidney wt., gm.	7.5	9.5	11.0	9.8	1.85	2.31
Kidney wt., gm./kgm. live wt.	6.8	7.7	9.1	9.5	0.77	0.82
Kidney crude protein, gm./kgm. live wt.	1.45	1.60	1.63	1.98	0.21	0.22
Kidney dry matter, %	23.6	23.3	21.0	23.1	1.8	2.0
Serum calcium mgm./100 ml.	10.4	65.9	33.8	26.9	5.3	4.6

** LSD denotes least significant difference (P = 0.05) by the "F" test (11) between (a) Groups A, B, C or (b) between Group D and any other group.*

*** NS denotes non-significance by "F" test of any group differences.*

TABLE V

EFFECTS OF THIOURACIL AND OF THYROXINE ON RESPONSE OF NUCLEIC ACIDS OF LIVER AND KIDNEY TO COMBINED TREATMENT WITH ESTROGEN AND ANDROGEN. EXPERIMENT 2. AVERAGE RESULTS

	Group A	Group B	Group C	Group D	LSD*	
	Treatment					
	Nil	ODB + TST	ODB + TST Thiouracil	ODB + TST Thyroxine	(a)	(b)
Liver						
DNAP mgm./100 gm. liver	29.5	21.9	22.1	24.0	2.9	3.2
DNAP mgm./kgm. live wt.	6.52	7.40	9.40	6.33	0.87	0.71
RNAP mgm./100 gm. liver	78.5	100.3	103.4	91.1	7.60	7.71
RNAP mgm./kgm. live wt.	17.8	33.9	42.4	24.1	2.8	2.1
Ratio RNAP/DNAP	2.75	4.60	4.72	3.83	0.49	0.43
Kidney						
DNAP mgm./100 gm. kidney	51.3	24.1	41.4	51.8	4.9	5.9
DNAP mgm./kgm. live wt.	3.45	3.22	3.78	4.85	0.33	0.37
RNAP mgm./100 gm. kidney	38.6	34.1	37.5	42.7	4.6	6.4
RNAP mgm./kgm. live wt.	2.60	2.59	3.43	3.97	0.23	0.24
Ratio RNAP/DNAP	0.75	0.81	0.91	0.82	0.07	0.07

** LSD denotes least significant difference (P = 0.05) by the "F" test (11) between (a) Groups A, B, C or (b) between Group D and any other group.*

TABLE VI

SUMMARY OF MAIN QUALITATIVE EFFECTS OF THIOURACIL AND OF THYROXINE ON THE RESPONSE OF THE IMMATURE PULLET TO TREATMENT WITH ESTROGEN PLUS ANDROGEN

	Experiment	Effect of estrogen plus androgen	Modification of the effect of estrogen plus androgen by	
			Thiouracil	Thyroxine
Oviduct weight	1	+ Large	Slightly depressed (NS)*	Slightly enhanced
	2	+ Large	Depressed	Depressed
Spleen weight	1	−	No effect	No effect
	2	− Slight ($P = 0.1$)	No effect	No effect
Heart weight	1 and 2	Nil	No effect	[Increased over controls]
Thyroid weight	1 and 2	Nil	[Increased over controls]	[Decreased below controls]
Liver weight, gm./kgm. live wt.	1 and 2	+ Large	Enhanced	Depressed
Liver DNAP, gm./kgm. live wt.	1 and 2	+ Slight	Enhanced	Depressed
Liver RNAP, gm./kgm. live wt.	1	+ Very large	Enhanced	No effect
	2	+ Very large	Enhanced	Depressed
Liver RNAP/DNAP	1 and 2	+ Very large	No effect	Depressed
Kidney weight, gm./kgm. live wt.	1	Nil	[Increased over controls]	[Increased over controls]
	2	+ Small	Enhanced	Enhanced
Kidney DNAP, gm./kgm. live wt.	1	Nil	No effect	[Increased over controls]
	2	Nil	[Increased over controls]	[Increased over controls]
Kidney RNAP	1 and 2	Nil	[Increased over controls]	[Increased over controls]
Kidney RNAP/DNAP	1 and 2	+ Slight (NS)	Enhanced	No effect
Serum calcium mgm./100 ml.	1 and 2	+ Large	Depressed	Depressed

**NS denotes non-significant by "F" test (11).*

(i) Oviduct weights:—In both experiments thiouracil slightly depressed the effect of ODB + TST on the oviduct, and this result attained significance in experiment 2. In a previous experiment (8) thiouracil was found to enhance the effect of ODB on the oviduct. Thyroxine enhanced the effect of ODB + TST on the oviduct in experiment 1, but depressed it in experiment 2, hence the results of the two experiments were at variance on this point. It will be recalled that Fleischmann and Fried (10) did not detect any appreciable effect of thyroxine on estrogen-induced hypertrophy of the chick oviduct.

(ii) Spleen weight:—ODB + TST depressed spleen weight significantly in experiment 1, but the effect only attained significance at $P = 0.10$ in experiment 2. However, previous work has shown (9, 14) that ODB reduces spleen weight, so that the effects in the present experiments are almost certainly real. The results do not provide any evidence that this effect of ODB + TST was modified by either thiouracil or thyroxine. Observations of this kind are hampered by the high variance of spleen weights in the pullet.

(iii) Heart weight:—It is evident from the results that heart weight was not affected by ODB + TST or by thiouracil, but that the thyroxine treatment led to pronounced cardiac hypertrophy.

(iv) Thyroid weight:—ODB + TST depressed thyroid weight slightly in experiment 1 and the effect almost attained significance at $P = 0.05$. This was in agreement with Keefe's (12) repeated observation in this laboratory of a slight depressant action of either ODB or ODB + TST on thyroid weight. In experiment 2, ODB + TST seemingly increased thyroid weight, but thyroid weights were unusually variable in this experiment and the effect was non-significant. Thiouracil produced the usual increase in thyroid weight and thyroxine the usual decrease, both effects being significant and of such a magnitude as to swamp any possible influence of ODB + TST.

(v) Liver weights:—ODB + TST increased liver weight per kgm. live weight in both experiments. This effect was enhanced by thiouracil and depressed by thyroxine in both experiments. These results are consistent with previous observations in respect of both thiouracil (8) and thyroxine (6).

The groups receiving thyroxine had a significantly lower percentage content of dry matter in their livers than the other three groups in both experiments. However, one may compare the results for liver dry matter and for crude protein most conveniently by expressing them as percentages of the relevant values for the control groups. The figures are set out in Table VII. Separate averages for each experiment are not shown, because the trends and significances of differences were similar in the two experiments. ODB + TST gave similar large percentage increases of liver weight and liver dry matter, but a distinctly smaller percentage increase of liver crude protein. This suggests that the increment of liver material was richer in lipid plus carbohydrate than the original liver material. Thiouracil enhanced the effect of ODB + TST on liver dry matter, and this was not due solely to enhanced deposition of lipid, for the effect on liver crude protein was enhanced also. In this connection it may be recalled that thiouracil has been observed to increase estrogen-induced protein deposition in the liver while depressing various estrogen-induced changes in the blood serum (8). The present observations suggest that such dissociated effects of thiouracil on deposition of protein in the liver and on serum calcium also occur when combined treatment with estrogen and androgen is the factor that evokes the changes. For, while thiouracil increased the deposition of protein in the liver, it decreased the hypercalcemia evoked by ODB + TST. Thyroxine did not simply reverse the effects of ODB + TST on the liver, for the livers of groups D displayed relatively little depression of the increase of crude protein evoked by ODB + TST, whereas the increase of liver dry matter was greatly reduced. This observation suggests that thyroxine had depleted liver lipid plus carbohydrate below the level obtaining in the control birds. In other words, the stimulation of catabolism by thyroxine fell preferentially on the lipid plus carbohydrate of the liver, but seems not greatly to have offset the anabolic effects of ODB + TST on liver nitrogen.

TABLE VII

EFFECTS OF THIOURACIL AND THYROXINE ON PERCENTAGE RESPONSES OF WEIGHT, DRY MATTER, CRUDE PROTEIN, AND MOISTURE OF LIVER AND KIDNEY TO TREATMENT WITH ODB + TST. AVERAGE RESULTS FOR BOTH EXPERIMENTS

	Groups			
	A	B	C	D
	Treatment			
	Nil	ODB + TST	ODB + TST Thiouracil	ODB + TST Thyroxine
Liver				
Gross wt. per kgm. live weight	100	152	187	121
Dry matter per kgm. live weight	100	150	181	108
Crude protein per kgm. live weight	100	130	173	125
Moisture per kgm. live weight	100	153	189	126
Kidney				
Gross wt. per kgm. live weight.	100	107	130	145
Dry matter per kgm. live weight	100	110	121	148
Crude protein per kgm. live weight	100	108	114	142
Moisture per kgm. live weight	100	102	133	144

(vi) Kidney weight:—ODB + TST did not affect kidney weight in experiment 1, and gave a slight significant increase in experiment 2. There is some other evidence that estrogen tends to increase kidney weight in the pullet (13), although the effect is small as compared with that on liver, and that TST tends to decrease kidney weight (13, 12). Thus, the net effect of ODB + TST given concurrently is presumably dependent on the amounts of each hormone given. Thiouracil increased kidney weight over that in the control group; and, in contrast with its effect on liver, thyroxine also increased kidney weight. This contrast is made clearer by expression of the data as percentages of the corresponding values for the control group (*vide* Table VII). Thiouracil depressed significantly the percentage of dry matter in the kidney, in spite of its positive effect on kidney dry matter per kgm. live weight. This explains why there is a relatively greater increase in kidney weight per kgm. live weight than in kidney crude protein per kgm. live weight (*vide* Table VII). Thyroxine seems to have given a larger kidney with the same content of dry matter and crude protein as in the control groups (*vide* Table VII). Moreover, the relative increases in the nucleic acids (*vide* Table VII) were of the same order as the relative increases of dry matter, crude protein, and moisture so far as the thyroxine-treated group was concerned.

(vii) Serum calcium:—The hypercalcemia evoked by ODB + TST was reduced by thiouracil and also by thyroxine, in agreement with previous results (10, 15, 16). It may be noted that thyroxine in amounts sufficient greatly to reduce hypercalcemia induced by ODB + TST does not appear necessarily to decrease the high calcium retention induced by treatment with

TABLE VIII

EFFECTS OF THIOURACIL AND THYROXINE ON PERCENTAGE RESPONSES OF NUCLEIC ACIDS OF LIVER AND KIDNEY TO TREATMENT WITH ODB + TST

	Groups			
	A	B	C	D
	Treatment			
	Nil	ODB + TST	ODB + TST Thiouracil	ODB + TST Thyroxine
Liver				
DNAP, mgm. per kgm. live weight	100	113	132	105
RNAP, mgm. per kgm. live weight	100	201	239	150
Kidney				
DNAP, mgm. per kgm. live weight	100	95	109	136
RNAP, mgm. per kgm. live weight	100	99	131	147

ODB + TST (6). Benoit and Clavert (1) have shown that injection of estrogenized ducks with thyroxine leads to more abundant formation of medullary bone, although this is less mineralized.

(viii) Liver and kidney nucleic acids:—ODB + TST slightly increased liver DNAP per kgm. live weight (though not significantly in experiment 1) and greatly increased liver RNAP in both experiments, these effects being in agreement with all similar observations in this laboratory (2, 7, 14, 15). Both effects were enhanced to a significant degree by thiouracil. Thyroxine did not appreciably affect liver DNAP in experiment 1, but depressed it in experiment 2; and in both experiments thyroxine significantly depressed the effects of ODB + TST on liver RNAP. ODB + TST greatly increased the ratio RNAP : DNAP in liver, again in agreement with previous work. This effect was not modified by thiouracil, whereas thyroxine significantly depressed the effect of ODB + TST on this ratio in both experiments. It is remarkable that thiouracil not only enhanced the increase of liver crude protein evoked by ODB + TST (*vide supra*), but also enhanced the increase of DNAP per kgm. live weight evoked by that treatment. Increased liver DNAP per kgm. live weight is possibly a consequence of mild hepatic hyperplasia. It has been shown by Clavert and Randavel (5) and by Clavert (3, 4) that treatment of pigeons with estrogen induces an hepatic hyperplasia demonstrable by histological methods.

In spite of the indications (*vide supra*) that ODB + TST increased kidney weight, this treatment did not increase either kidney DNAP or RNAP, and the slight increase of the ratio kidney RNAP : DNAP did not attain significance at $P = 0.05$. It is evident that the effects, if any, of ODB + TST on the kidney nucleic acids were slight in comparison with the effects on liver nucleic acids. It is remarkable, therefore, to note that thiouracil significantly increased kidney RNAP per kgm. live weight, but not kidney DNAP per kgm.

live weight as compared with the values for either group A (control) or Group B (ODB + TST only), and this was true of both experiments. Kidney DNAP and RNAP per kgm. live weight were both significantly greater in group D (thyroxine) than in group A (control) or Group C in both experiments. Moreover, it is clear that this was not simply a consequence of the effect of thyroxine on live weight.

The effects of the various treatments on liver and kidney nucleic acids are summarized in Table VIII, where the results have been stated as percentages of the values for the control group. The salient features are the absence of any effect of ODB + TST on kidney as compared with liver; the absence of an increase of liver DNAP in presence of thyroxine, whereas kidney DNAP was greatly increased; and the depression of the effect of ODB + TST on liver RNAP by thyroxine, whereas this treatment, if anything, gave a greater increase in kidney RNAP than did thiouracil.

In previous work (12) it has been found that 0.75% thiouracil in the diet not only increased the effect of estrogen on liver weight per kgm. live weight, but also of itself increased liver weight per kgm. live weight. It is likely, therefore, that the effects of thiouracil on liver nucleic acids in the present experiment represent a superimposition of its effects on positive effects of the treatment with gonadal hormones; whereas the effects of thiouracil on kidney nucleic acids may represent the effect of this substance per se, since the treatment with gonadal hormones did not appreciably affect the kidney content of nucleic acid. However, confirmation of this suggestion must await the results of further experimentation.

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INFLUENCE OF EXPOSURE TO COLD AND ASCORBIC ACID ADMINISTRATION ON THE WEIGHT AND THE METABOLIC ACTIVITY OF THE ADRENAL GLAND OF HYPOPHYSECTOMIZED RATS GIVEN ACTH[1]

By L.-P. Dugal, A. DesMarais, and P. M. Gagnon

Abstract

Modification of the response of the adrenal gland to ACTH was observed in hypophysectomized rats exposed to cold with or without ascorbic acid treatment. Changes in weight and metabolic activity of the gland were used to measure the adrenal response. All comparisons were made between the left and the right adrenal of each animal before and after treatment. The results obtained show that cold stress increases the response of the adrenal to a standard dose of two units of ACTH; they also indicate that ascorbic acid administration increases so much the responsiveness of the adrenal gland to ACTH at room temperature that such a response is not enhanced by exposure to cold.

It is generally agreed that there is an increase of adrenal activity during exposure to cold (or to any stress), and that such a hyperactivity, at least as far as the adrenal cortex is concerned, is mediated through the anterior pituitary. The purpose of the present experiment was to find out if cold itself had no direct effect on the adrenal activity, provided there was a minimum amount of ACTH available, and whether ascorbic acid treatment would modify this effect of cold.

Experimental

Two series of experiments were done, the first one to study the effects of cold, the second to find out the influence of ascorbic acid.

1. *First Series: Cold Exposure Alone*

This experiment was performed on 178 hypophysectomized male albino rats of the Wistar strain, weighing 110 to 140 gm. They were all fed the diet of Shaw and Greep (11) ad libitum. Eight days after hypophysectomy, their left adrenal was removed and weighed wet. Starting immediately after this operation, they all received two units of ACTH a day. Hence, any difference in adrenal weight between the two groups could not be ascribed to the amount of ACTH injected, since it was the same, but rather to a more or less efficient utilization under different conditions of environment. Eighty-four of these animals were kept at room temperature (22° C.), and the rest, 94, were exposed to a temperature of 14 ± 2° C. Cold exposure was started 48 hr. after the left adrenalectomy. Six days later, the right adrenal of all animals of both groups was removed, weighed wet, and comparisons were made between the weight of the right adrenal of each animal of each group and the weight of the left adrenal of the same animal.

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Contribution from the Department of Experimental Physiology, Faculty of Medicine, Laval University, Quebec.
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2. *Second Series: Cold and Ascorbic Acid*

The experimental arrangement was exactly the same, except that the two groups, room temperature and cold exposed, were subdivided in sodium-ascorbate (150 mgm. intraperitoneal daily) and sodium bicarbonate (same amount of sodium) treated animals, the treatment being initiated immediately following the left adrenalectomy. In this experiment, the adrenals were not only weighed, but also assayed for their succinic dehydrogenase activity according to the method of Perry and Cumming (10). Comparisons were made between the left and the right adrenal of each animal, as in the previous experiment.

Results

The results are shown in Table I, and in Tables II and III, corresponding respectively to the first and second series of experiments.

Table I gives the average weights of the adrenals and thymus for both groups. The increase in adrenal weight is statistically significant only for the group exposed to cold, and the difference of the increase in adrenal weight between the two groups is also highly significant. Also significant is the difference in thymus weight, the group exposed to cold (the one with a higher increase in adrenal weight) showing the smaller average weight for the thymus.

TABLE I

EFFECT OF EXPOSURE TO COLD ON THE ADRENAL WEIGHT OF HYPOPHYSECTOMIZED RATS RECEIVING ACTH

Temperature	Number of animals	Left adrenal weight, mgm.	Right adrenal weight, mgm.	Difference, mgm.	p	Thymus, mgm.	p
Cold	94	7.85	8.96	1.11 ± 0.13	< 0.001	219.6	< 0.01
Room	84	7.87	8.15	0.28 ± 0.13		243.7	

Table II shows the results obtained for the metabolic activity of the adrenal, either expressed as absolute (per adrenal) or relative (per unit weight of tissue) activity: they are in the same direction and also statistically significant. They indicate that the adrenals of hypophysectomized rats have a greater activity after exposure of the animals to cold. It can also be seen that ascorbate administration greatly increases the metabolic activity of the adrenal at room temperature, and that no further enhancement is observed in the cold when the animals are treated with sodium ascorbate.

Table III gives the average increases in weight between the left and the right adrenals of the animals, in the second series of experiments. Although the effect of cold alone, here, is not statistically significant, we can see that the results are parallel to those obtained on the metabolic activity.

TABLE II

ABSOLUTE AND RELATIVE DEHYDROGENASE ACTIVITY

Treatment	Number of animals	Left adrenal, TTC μgm.	Right adrenal, TTC μgm.	Difference, TTC μgm.	Probability against	
					Bic., room temp. °C.	Controls, room temp. °C.
Absolute dehydrogenase activity						
Bicarbonate—room temp.	25	2.97	5.41	2.44 ± 1.00	—	—
Ascorbate—room temp.	16	2.14	12.89	10.75 ± 2.49	< 0.01	—
Bicarbonate—cold	17	1.40	8.12	6.72 ± 2.08	< 0.01	< 0.01
Ascorbate—cold	11	1.15	10.77	9.62 ± 2.03	< 0.01	< 0.80
Relative dehydrogenase activity						
Bicarbonate—room temp.	25	54.42	87.84	33.4 ± 12.5	—	—
Ascorbate—room temp.	16	43.12	154.04	110.9 ± 18.8	< 0.01	—
Bicarbonate—cold	17	25.98	103.42	77.4 ± 14.9	< 0.05	< 0.05
Ascorbate—cold	11	22.72	135.87	113.1 ± 19.1	< 0.01	< 0.90

TABLE III

INCREASE IN ADRENAL WEIGHT FROM LEFT TO RIGHT ADRENAL AFTER EXPOSURE TO COLD WITH OR WITHOUT ASCORBIC ACID

Treatment	Number of animals	Adrenal weight increase, mgm.	Probability against	
			Bic., room temp. °C.	Controls, room temp. °C.
Bicarbonate—room temp.	25	1.31 ± 0.33	—	—
Ascorbate—room temp.	16	3.61 ± 0.58	< 0.01	—
Bicarbonate—cold	17	2.16 ± 0.52	< 0.20	< 0.20
Ascorbate—cold	11	3.14 ± 0.54	< 0.01	< 0.01

Discussion

In the last years, particularly from the work of Ingle (6, 7, 8, 9), the permissive action of the cortical hormones towards certain metabolic processes has become more and more prominent, and the same phenomenon has been found to extend to other hormones, for instance to the thyroid hormones which have a permissive action on the calorigenic effect of adrenalin (12).

Conversely, certain conditions or treatments have been shown to exert some influence on the sensitivity of target organs or reactions to the action of an invariable amount of hormones. Herlant (4, 5) has demonstrated that exposure to cold sensitizes the thymus towards the involutive effect of the cortical hormones; similarly, Dugal and Thérien (2, 3) and DesMarais and Leblanc (1) have shown that ascorbic acid administration enhances the sensitivity of the adrenal gland towards adrenocorticotrophic hormone.

The present results are a further demonstration of the permissive action of hormones. Exposure to cold increases the sensitivity of the adrenal towards ACTH, as may be seen from the increase of the weight of the gland and of its secretory activity, in so far as the metabolic activity of the adrenal can be used as an index of its secretory activity. Ascorbic acid increases the responsiveness of the adrenal gland to ACTH at room temperature to such a degree that the response is not enhanced by exposure to cold.

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THE *IN VITRO* ADRENAL RESPONSE TO CORTICOTROPHIN SUBTYPES

AN IMPROVED ASSAY DESIGN[1]

By K. W. McKerns and E. Nordstrand

Abstract

An eight point assay design for assessment of corticotrophin potency in terms of any standard corticotrophin is described. Each point can be repeated four times by using littermate rat adrenal tissue. The response of isolated adrenal tissue to corticotrophin stimulation is measured in terms of the corticoids produced. As many as three unknown samples may be measured in terms of one standard or compared with one another. Three subtypes of corticotrophin have been assessed in one assay design. They are acid extracted crude corticotrophin (represented by the original U.S.P. Provisional Reference Standard), glacial acetic acid extracted, and oxycel purified corticotrophin. All these corticotrophins showed the same dose–response characteristics and relative potencies in agreement with the Sayers ascorbic acid depletion assay by the intravenous route. When three different samples were assayed in one design of 32 rats the average lambda was 0.18, and the limits of error for each sample were approximately ± 45%.

Introduction

We have previously described a four point assay design for the assessment of corticotrophin potency in terms of the U.S.P. Provisional Reference Standard (3). The increased steroid output of an *in vitro* adrenal gland system incubated with added corticotrophin is the basis of the method. Saffran and Schally have also proposed an *in vitro* corticotrophin assay of different design (5). We have incorporated their quartered-adrenal technique in this assay.

This paper presents an eight point assay design that enables three unknown samples to be measured in terms of a standard. As alternatives, a duplicate four point assay design or two unknown samples compared with a double two point standard may be run. It is also of interest to examine by this *in vitro* system, directly at the adrenal level, several types of corticotrophin now being proposed as International and U.S.P. Reference Standards. This has been done for crude acid extracted (the original U.S.P. Provisional Reference Standard), the glacial acetic acid extracted type of Payne *et al.* (4), and the oxycel purified type of Astwood *et al.* (1). These materials have been assayed within one assay design such that their dose–response relationships, parallelism of response, and related potency can be directly assessed. A comparison of the potencies obtained by this *in vitro* assay is made with the potencies obtained by the Sayers ascorbic acid depletion method (6) by both the intravenous and subcutaneous routes. The glacial acetic and oxycel purified materials are clinically more active than would be expected from their Sayers ascorbic acid depletion assay by the intravenous route.

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Contribution from the Research and Development Laboratories, Canada Packers Ltd., Toronto, Ontario.

Materials and Methods

Eight litters of young female rats of four littermates each are used per assay. Each adrenal gland of the pair from one rat is quartered, and a piece placed in each of eight sectors on a buffer moistened filter paper. This distribution of adrenal tissue is repeated for each of eight rats—one from each litter. Each of the eight sectors thus contains eight pieces of adrenal tissue representing a quarter adrenal from either a left or right adrenal of each of the eight rats. The tissue in a sector (approximately 25 mgm.) is weighed and transferred to one of eight small flat-bottomed tubes containing 1 ml. of Krebs Ringer bicarbonate buffer. These eight tubes constitute the "doses" across of the assay design. This distribution of adrenal tissue is repeated three times with the littermates, making the four "series" of the design. Two series are usually handled in one day and the second half completed on the following day.

The adrenal pieces are incubated at 37° C. in equilibrium with 95% oxygen, 5% carbon dioxide for one hour as previously described (3). The buffer is aspirated from the gland tissue and 1 ml. fresh buffer containing corticotrophin is added for an additional two hours. This buffer is removed and extracted with 2 ml. methylene chloride by shaking approximately 100 times. The aqueous layer is aspirated off. Anhydrous sodium sulphate (5–10 mgm.) is added to the methylene chloride which can then be poured into 1.5 ml. silica cells. The estimation of adrenal cortical output by measuring the optical density at 240 mμ with appropriate corrections for interfering substances has been described (2, 3).

The dose level of added corticotrophin in all experiments was adjusted to the adrenal tissue weights so that the lower dose was 0.015 and the upper dose 0.045 I.U./100 mgm. adrenal tissue. The corticoid value for each flask is expressed in terms of gamma cortisone acetate per ml. extraction solvent and may be multiplied by two to obtain gamma corticoids per 100 mgm. adrenals.

The corticotrophin samples analyzed in this study are:

1. #269—a glacial acetic extracted preparation proposed as a second U.S.P. and World Health Organization standard.
2. R216–129—an oxycel purified glacial acetic extracted corticotrophin having a subcutaneous to intravenous potency ratio of approximately 3 : 1 by the Sayers ascorbic acid depletion assay.
3. The first U.S.P. Provisional Reference Standard.

Results

The corticoid responses for three corticotrophin samples and the U.S.P. Provisional Reference Standard compared together in one assay design are given in Table I.

The corticotrophins were assayed at assumed potencies of 0.005, 1.0, and 20.0 I.U./mgm. in assays *a* and *b* for samples Standard, #269, and R216-129 respectively. In assay *c* the assumed potencies were 0.005, 0.70, and 18.0 I.U./mgm.

TABLE I

RELATION BETWEEN DOSE AND CORTICOID OUTPUT FOR THREE DIFFERENT TYPES OF CORTICOTROPHIN

	Standard		#269		R216-129		Standard		
Assay No.	L	H	L	H	L	H	L	H	Totals
a.	21.39	26.35	15.00	20.57	18.10	21.52	16.90	26.64	166.47
	19.96	29.00	17.42	24.61	21.11	25.63	20.27	26.25	184.25
	21.28	21.27	14.57	20.55	22.81	22.38	19.38	24.35	166.59
	17.01	18.45	14.78	20.17	16.69	25.24	14.77	22.97	150.08
Totals	79.64	95.07	61.77	85.90	78.71	94.77	71.32	100.21	
b.	18.31	26.07	17.66	25.45	24.29	25.85	17.85	29.73	185.21
	18.31	29.66	22.89	24.81	20.12	24.37	22.37	31.59	194.12
	18.85	19.04	17.14	23.70	18.94	22.72	22.81	26.83	170.03
	30.40	33.51	19.61	29.13	18.83	31.82	25.76	35.62	224.68
Totals	85.87	108.28	77.30	103.09	82.18	104.76	88.79	123.77	
c.	19.63	26.95	17.98	27.54	20.08	29.65	19.19	28.52	189.54
	24.53	33.21	26.55	30.09	25.27	37.78	27.04	32.74	237.21
	19.74	22.35	16.03	24.57	23.60	31.75	18.88	28.43	185.35
	23.86	30.56	25.27	36.56	27.94	31.80	21.75	33.50	231.24
Totals	87.76	113.07	85.83	118.76	96.89	130.98	86.86	123.19	

Note: The doses across were in each case 0.015 for the low (L) and 0.045 I.U./100 mgm. for the high (H). The results are expressed as gamma corticoids per 50 mgm. adrenals per two hours.

TABLE II

ANALYSIS OF VARIANCE FOR THE DATA OF TABLE I

Assay No.	Nature of variation	Degrees freedom	Sum of squares	Mean square	*F* ratio
a.	Total	31	463.85	—	—
	Series	3	73.03	24.34	5.69
	Doses	7	301.05	43.01	10.06
	Concentrations (average slope)	1	223.19	223.19	52.21
	Preparations	3	61.94	20.65	4.83
	Conc. × prep. (slope difference)	3	15.93	5.31	1.24
	Error	21	89.77	4.275	
b.	Total	31	831.67		
	Series	3	199.02	66.34	7.04
	Doses	7	434.84	62.12	6.59
	Concentrations (average slope)	1	349.51	349.51	37.10
	Preparations	3	72.32	24.11	2.56
	Conc. × prep. (slope difference)	3	13.09	4.363	0.463
	Error	21	197.81	9.420	
c.	Total	31	960.75		
	Series	3	276.90	92.30	18.59
	Doses	7	579.61	82.80	16.68
	Concentrations (average slope)	1	517.29	517.29	104.21
	Preparations	3	53.73	17.91	3.61
	Conc. × prep. (slope difference)	3	8.59	2.86	0.58
	Error	21	104.24	4.964	

The three samples are evaluated in terms of the standard:

Assay No.	$\bar{b}$ = mean slope	s = standard error	$\lambda = s/b$
a.	11.07	4.275	0.187
b.	13.85	3.07	0.222
c.	16.85	2.228	0.132

They show the following relative potencies and limits:

Assay No.	#269	R216-129	Standard
a.	0.495 I.U./mgm. (0.807 to 0.304)	14.53 I.U./mgm. (22.84 to 9.238)	0.0046 I.U./mgm. (0.0072 to 0.0030)
b.	0.7513 I.U./mgm. (1.283 to 0.4399)	17.22 I.U./mgm. (29.20 to 10.15)	0.0073 I.U./mgm. (0.0126 to 0.00426)
c.	0.7462 I.U./mgm. (1.021 to 0.546)	28.57 I.U./mgm. (39.62 to 20.61)	0.00619 I.U./mgm. (0.00849 to 0.00451)

In Table III the U.S.P. Provisional Reference Standard is run against R216-129 at 18 I.U./mgm.

TABLE III

	Standard		R216-129		Standard		R216-129		Total
	L	H	L	H	L	H	L	H	
	21.66	31.06	20.85	35.50	23.41	29.14	28.90	30.85	221.37
	22.79	30.76	19.56	22.02	21.33	32.17	16.27	26.56	191.46
	20.68	24.48	25.37	29.75	18.96	23.96	19.57	24.51	187.28
	15.27	26.14	16.69	27.30	16.21	22.81	18.07	29.09	171.58
Total	80.40	112.44	82.47	114.57	79.91	108.08	82.81	111.01	—

TABLE IV

ANALYSIS OF VARIANCE FOR THE DATA OF TABLE III

Nature of variation	Degrees freedom	Sum of squares	Mean square	F ratio
Total	31	865.52	—	—
Series	3	162.35	54.116	5.50
Doses	3	462.24	154.080	15.65
Concentrations (average slope)	1	453.83	453.83	46.09
Preparations	1	3.14	3.14	0.32
Conc. × prep. (slope difference)	1	0	0	0
Error (*a*)	4	5.25	—	—
Error (*b*)	21	240.93	—	—
Error (total)	25	246.18	9.847	—

$b = 15.79$; $s = 3.138$; $\lambda = 0.199$. Relative potency and limits R216-129 = 19.73 I.U./mgm. (31.6 to 12.29).

TABLE V

WEIGHTED MEAN POTENCIES OF THE *in vitro* ASSAY DATA COMPARED WITH THE SAYERS ASSAY BY THE INTRAVENOUS (I.V.) AND SUBCUTANEOUS (S.C.) ROUTES
(Potencies are expressed as I.U./mgm.)

Preparation	Sayers assay			*In vitro* assay	
	Activity		Limits error	Activity	Limits error
	I.V.	S.C.			
#269	0.70	1.08	± 10%	0.65	+ 25 − 20.5%
R216-129	21.3	60.0 (approx.)	± 10%	20.5	+ 24 − 18.5%
U.S.P. Standard	0.005	0.005	± 10%	0.0059	+ 25.3 − 20.2%

Discussion

There is no difference between the potencies obtained for various subtypes of corticotrophin by this assay design and the previous design (3), since all potencies are relative to a standard corticotrophin. However this new arrangement of assay design effects a considerable saving in the number of animals required to achieve comparable limits of error. Three unknown samples can be assayed with 32 rats for an average lambda of 0.18 and limits of error of approximately ± 45%, which is excellent for this type of assay.

The degrees of freedom left to the error calculation have been increased from 9 in the old design to 21 in the three-unknown design. Of course the number of degrees of freedom for error greatly influences the limits of error of the assay where limits are equal to $\log R \pm t(S_{\log R})$.

If each of the four dose lines across were calculated separately, the lambdas for each would be approximately equal to the total lambda of the complete design but the degrees of freedom for error would drop to three, with a very large increase in the limits of error. Similarly, assays based on two dose lines would have only 6 degrees freedom, or 12 degrees for two half assays compared with the 21 degrees freedom for the complete design.

A design based on only one unknown gives only a slight increase in the degrees of freedom for error (25 degrees—Table IV). A further arrangement is possible based on two unknowns and a duplicate standard.

The potencies obtained by this *in vitro* assay method are in close agreement with the potencies obtained by the Sayers ascorbic acid depletion method using the intravenous route (Table V).

The analyses of variance for the response data (Table II) show that there are no significant differences in slope between the different subtypes of corticotrophin (8) assayed. The average slopes between the low and high doses are highly significant for all preparations.

This parallelism of dose response for the so-called subtypes of corticotrophin (8) by an *in vitro* adrenal system is again in agreement with Sayers' method

by the intravenous route. It is unlike the lack of parallelism found by Thompson and Fisher (7) between the subtypes of corticotrophin when assayed by *in vivo* subcutaneous methods.

No differences were demonstrated at the adrenal level between subtypes or corticotrophins of different preparative history and presumably different chemical structure. This must mean that differences in rates of absorption from an intramuscular or subcutaneous depot or variations in extravascular stability account for the intravenous–subcutaneous potency differences.

Acknowledgments

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THE INFLUENCE OF POTASSIUM ION UPON GLUCOSE UPTAKE AND GLYCOGEN SYNTHESIS IN THE ISOLATED RAT DIAPHRAGM[1]

By D. W. Clarke

Abstract

The amounts of glucose taken from a medium, and the amounts of glycogen synthesized, by rat hemidiaphragms were studied under various conditions. High concentrations of potassium ion inhibited the glucose uptake and there was also a reduced net glycogen synthesis. Glycogen breakdown was probably not increased by high potassium ion concentration. The effect of potassium was most marked when conditions were such that one would ordinarily expect a considerable glucose uptake or glycogen synthesis. The action of insulin was not peculiarly susceptible to potassium ion inhibition.

It was shown by Stadie and Zapp in 1947 that the presence of K^+ ions in the incubation medium reduced the glycogen synthesis in the isolated rat diaphragm in the presence of glucose and insulin. High concentrations of K^+ reduced, as well, the net glycogen synthesis without insulin. Similar results were reported by Tuerkischer and Wertheimer (9) who found that K inhibited the synthesis of glycogen from glucose. These results should be compared with those of Hastings (3, 2, 6) which indicated that in liver slices, K^+ promoted the synthesis of glycogen. Stadie and Zapp (8) measured only the net glycogen synthesis and assumed that a decrease in glycogen was due to an inhibition of those reactions which synthesized glycogen. Tuerkischer and Wertheimer also measured net glycogen synthesis, with the same assumptions which Stadie used. In addition, they showed that there was a net glycogenolysis (i.e. the final glycogen in the tissue was lower than the initial glycogen) in the presence of high concentrations of K^+, and concluded that K^+ in high concentrations favored glycogenolysis. Any change in glycogen, however, is not necessarily a result of a single reaction, but may result from a change in either or both of the processes of glycogenesis or glycogen breakdown. It was felt that further information on the action of K^+ in the carbohydrate metabolism of muscle might be gained if, instead of measuring only glycogen synthesis, both glucose uptake and glycogen synthesis were measured under different conditions of treatment with insulin, glucose, and Na^+ or K^+ ions. A comparison of the action of K^+ upon the "insulin effect", and the influence of K^+ upon the glucose uptake and glycogen synthesis of a muscle not treated with insulin also seemed desirable.

"Insulin effect" is defined as the difference between the amount of glycogen synthesized by a muscle treated with insulin, and the amount of glycogen synthesized by a similar, untreated muscle. The amounts of glycogen are

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Contribution from the Department of Physiology, University of Toronto, Toronto, Ontario.

expressed as milligrams of glucose per gram of tissue. Similarly, the difference between the amount of glucose taken up, per gram of tissue, by an insulin treated muscle and a non-treated muscle is defined as the "insulin effect" on glucose.

In the first series of experiments, diaphragms from fed or fasted animals were incubated with glucose in a buffered medium, with or without the addition of insulin, and the glucose uptakes and glycogen syntheses were measured. In the second series the diaphragms were similarly incubated, but in media which did not contain glucose. This afforded a means of studying the glycogenolytic effects only.

Experimental

Male Wistar rats, weighing from 150 to 250 gm., were fasted for 20 hr. prior to the experiment, or else allowed to eat ad libitum until they were used. They were decapitated, and the diaphragms were removed and placed in cold media (of the same ionic composition as that subsequently used in the experiment, but without glucose) for 15 min. before incubation in the appropriate media. This preliminary soaking has been reported by Brown *et al.* (1) to give an increased glucose uptake.

Two different media were used. The "sodium" medium had the composition suggested by Stadie (8) of 0.04 *M* NaCl, 0.087 *M* Na_2HPO_4, 0.005 *M* $MgCl_2$, pH 6.80. The "potassium" medium had the sodium salts replaced by equal concentrations of potassium salts.

After preliminary soaking, the diaphragms were removed from the medium and the central portion was cut away. From one third to one quarter of each of the remaining portions was removed, and these small segments were quickly blotted, weighed together, and put into hot KOH for a determination of their glycogen content according to the procedure of Good, Kramer, and Somogyi (4). The remaining "halves" were placed in 50-cc. glass-stoppered Erlenmeyer flasks which contained 2 cc. of medium, with glucose added to a final concentration of 200 mgm. %. To one of the flasks, two units of insulin had been added.

The stoppered flasks were shaken at 37° for one hour, at the end of which time the diaphragms were removed and glycogen determinations done on whole portions according to the procedure mentioned above. A 1 ml. aliquot of the medium was used for the determination of glucose, according to the method of Somogyi (7). Control aliquots were removed from a flask which contained a sample of the original medium–glucose mixture, but which had been incubated without any tissue.

Results

In the discussion of the results, the following points should be borne in mind:

(a) The results obtained by using the "sodium" medium are arbitrarily used as a standard, and statements regarding inhibition or acceleration of an

effect in this experiment should be considered as meaning an inhibition or acceleration compared to the effect under similar conditions but using the "sodium" medium.

(b) The ionic composition of the media is non-physiological. Conclusions drawn from these experiments should be considered only as applying to the peculiar conditions existing, and should be extended to physiological conditions with caution. These media were chosen because of their similarity to a medium which has been fairly widely employed in recent years.

Two series of experiments were performed. In Series I the diaphragms were incubated in media with glucose. In Series II there was no glucose in the media.

A summary of the results of the experiments in Series I is shown in Table I, Figs. 1 and 2. Values are expressed as mgm. glucose per gm. of tissue. It will be apparent that several sets of differences may be found. For simplicity, those differences which result from experiments on fasted animals, and those on fed animals, are shown in Table I. Those differences which come about as a result of experiments in which the effect of K^+ was studied are shown in Figs. 1 and 2, under appropriate columns. The original values from which these differences are obtained are of course those shown in Table I.

TABLE I

GLUCOSE UPTAKE AND GLYCOGEN SYNTHESIS

	Sodium medium				Potassium medium			
	A	B	C	D	A[1]	B[1]	C[1]	D[1]
Glucose uptake—mgm./gm. of tissue								
No insulin in medium	4.39 ±0.29	2.70 ±0.42	1.69 ±0.54	0.01	2.64 ±0.38	2.65 ±0.49	0.01 ±0.63	N.S.
Insulin in medium	6.29 ±0.54	5.39 ±0.32	0.90 ±0.60	N.S.	4.20 ±0.63	3.86 ±0.38	0.34 ±0.72	N.S.
Insulin effect*	1.90 ±0.52	2.69 ±0.50	0.79 ±0.72	N.S.	1.56 ±0.40	1.21 ±0.45	0.35 ±0.60	N.S.
N	8	10			8	9		
Glycogen synthesis—mgm./gm. of tissue								
No insulin in medium	0.07 ±0.18	−0.81† ±0.22	0.88 ±0.29	0.01	0.08† ±0.16	−0.42 ±0.47	0.50 ±0.52	N.S.
Insulin in medium	0.78 ±0.19	0.07 ±0.32	0.71 ±0.40	N.S.	0.25 ±0.10	−0.30† ±0.38	0.55 ±0.42	N.S.
Insulin effect*	0.71 ±0.18	0.88 ±0.19	0.17 ±0.27	N.S.	0.17 ±0.13	0.12 ±0.22	0.05 ±0.26	N.S.

Column A—Results obtained with fasted animals ± standard error of mean.
Column B—Results obtained with fed animals ± standard error of mean.
Column C—Difference due to feeding—i.e. (A) − (B), ± standard error of difference.
Column D—P value of difference (C).
*Note: * "Insulin effect" = glucose uptake (or glycogen synthesis) in presence of insulin less glucose uptake (or glycogen synthesis) without added insulin.*
† Negative values mean that there was less glycogen per mgm. of tissue after incubation than there was at the start of the incubation.

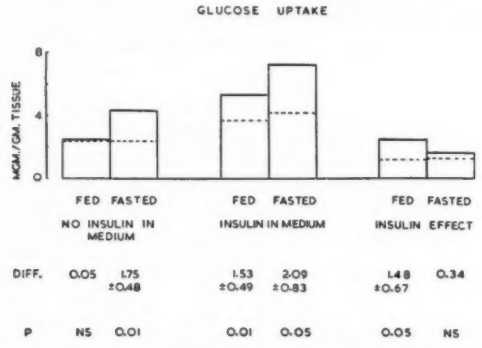

FIG. 1. Glucose uptake in rat hemidiaphragm. Height of bars with solid tops shows effect of Na^+ medium. Height of bars with dotted tops shows effect of K^+ medium. Differences are given with standard error of difference. Where no standard errors are given, it is obvious by inspection that there is no significant difference between the values concerned.

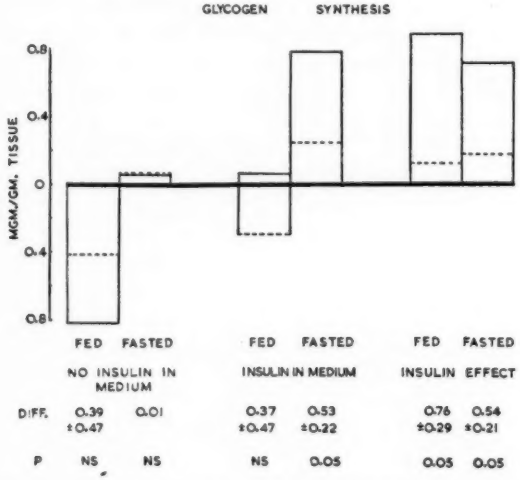

FIG. 2. Glycogen synthesis in rat hemidiaphragm. Symbols and figures have the same meaning as in Fig. 1.

Series I

A. Fasted Animals

I. *Glucose uptake.*—If the diaphragm has not been treated with insulin, potassium exerts a significant effect in reducing the amount of glucose which is removed from the medium by the tissue (Columns A, A^1, Row 1, Table I). If the diaphragm has been treated with insulin, there is the usual increase in glucose uptake, and potassium significantly reduced this increase (Columns A, A^1, Row 2). In both instances, however, the amount of reduction by

potassium is approximately the same, so there is no significant influence of potassium upon the "insulin effect" (Columns A, A^1, Row 3).

II. *Glycogen synthesis.*—In diaphragms which have not been treated with insulin, there is a small amount of glycogen synthesis, which is unaffected by the substitution of potassium ions for sodium ions in the medium (Columns A, A^1, Row 4). The addition of insulin gives an increase in glycogen synthesis, and the amount of this increase is significantly reduced by potassium ion (Columns A, A^1, Row 5). There is, consequently, a significant difference in the "insulin effect" upon glycogen synthesis as a result of incubation in the "potassium" medium (Columns A, A^1, Row 6).

B. Fed Animals

I. *Glucose uptake.*—With diaphragms which have had no insulin treatment, potassium does not appear to inhibit glucose uptake (Columns B, B^1, Row 1). If insulin is added to the medium, there is found the usual increase in glucose uptake (Columns B, B^1, Row 2). The amount of this increase is significantly reduced if the diaphragm is incubated in the "potassium" medium, and therefore a significant effect of potassium upon the "insulin effect" is noted (Columns B, B^1, Row 3).

II. *Glycogen synthesis.*—Under the conditions of the experiment, there is glycogen breakdown in a diaphragm incubated in the "sodium" medium, without insulin, even if glucose is present. The amount of this breakdown is reduced if the incubation is performed in the "potassium" medium (Column B, B^1, Row 4). If insulin is added, there is an increase in glycogen synthesis, which in the case of the "sodium" medium is sufficient to give a *net* increase in glycogen. If the potassium medium is used, the addition of insulin does not significantly alter the amount of glycogen breakdown (Columns B, B^1, Row 5). In both these cases the amount of reduction caused by the potassium ion is not statistically significant. It seems possible, though, that this lack of significance is due to a relatively high standard deviation, and it is unlikely that there is no K^+ effect. If the "insulin effect" is considered, however, the differences are now sufficiently great that the effect is significant, and the potassium medium is seen to inhibit the "insulin effect" upon glycogen synthesis (Columns B, B^1, Row 6).

C. A Comparison Between Fed and Fasted Animals

The effects of using animals which had been fed ad libitum until the time of the experiment, instead of animals which had been fasted prior to the experiment, are also shown by comparing Columns A and A^1 with Columns B and B^1. In most instances there was no significant difference between the results obtained with diaphragms from fed animals and those obtained with diaphragms from fasted animals. It was noted, however, that fasting of the animals significantly increased the glucose uptake and glycogen synthesis in diaphragms which were not exposed to insulin. These results are in accord with the finding of Hansen, Rütter, and Samuels (5).

Series II

D. Glycogen Breakdown in Sugar-free Media

In this series of experiments the tissues were incubated in the "sodium" or "potassium" medium as before, but no glucose was added. Measurements of glycogen contents before and after incubation gave an indication of the degree of glycogen breakdown and how it had been affected by various factors in this experiment. The results are shown in Table II.

TABLE II

GLYCOGEN BREAKDOWN IN SUGAR-FREE MEDIA*, SERIES II

Sodium			Potassium		
Initial glycogen	Final glycogen	Difference	Initial glycogen	Final glycogen	Difference
Fed animals					
2.81 ± 0.39	1.60 ± 0.23 (With insulin)	1.21 ± 0.23	3.31 ± 0.40	2.07 ± 0.23 (With insulin)	1.24 ± 0.28
	1.87 ± 0.42 (No insulin)	0.94 ± 0.40		2.24 ± 0.34 (No insulin)	1.07 ± 0.26
Fasted animals					
1.03	0.29† (With insulin)	0.74			
	0.27† (No insulin)	0.76			

** All values are expressed in mgm. glucose/gm. of tissue, ± standard error of mean or standard error of difference.*

† Standard errors were not calculated for these values, since inspection shows that there is no significant difference in them.

With fed animals there was no significant difference in the amounts of glycogen breakdown which occurred in the different media, with or without insulin. With fasted animals there was a slightly smaller amount of breakdown than with fed animals. Insulin had no effect.

Discussion

The general trend of the results shows that the potassium exerts an inhibitory effect upon glucose uptake, as well as upon glycogen synthesis. This suggests that the lowered net glycogen synthesis might come about as a result of decreased glucose uptake, and it will be suggested later that the entire inhibitory effect lies in this action upon glucose uptake, and that there is no increased glycogen breakdown in the "potassium" medium. The greatest inhibitory effect of potassium seems to occur when conditions are such (through fasting, or by the action of insulin) that there would ordinarily be a high glucose uptake or glycogen synthesis.

The effect of potassium, or of insulin treatment, upon the extent of glycogen breakdown is studied in Series II experiments (see Table II). Diaphragms were incubated in media which did not contain sugar, but which were otherwise the same as those in Series I. The amount of glycogen loss, as a result of this incubation, was measured. From the data shown in Table II, it is seen that the amount of glycogen breakdown was not significantly altered by using K^+ as the main cation, nor did the presence of insulin give any significant change. Since it is likely that very similar conditions, as far as glycogen breakdown is concerned, exist in the Series I experiments, we therefore conclude that any measurable change in glycogen must have come about as a result of a change in the reactions of glycogen synthesis.

If, from the various sets of experiments of Series I, the amounts of glycogen synthesized are plotted against the amounts of glucose taken up by the tissues, a graph as shown in Fig. 3 results. The slope of the plotted regression line is highly significant ($p = 0.01$) Points derived from runs in which insulin was used, or in which the "potassium" medium was used, do not fall consistently on one side or the other of the line. This suggests that the effect of these variables (i.e. insulin treatment or the use of the "potassium" medium) is only upon those reactions which are concerned with glucose uptake. Inhibition of the reactions leading from glucose-6-phosphate to glycogen would probably result in a decrease in glucose uptake, but since some glucose could still go to lactic acid or pyruvic acid, it might be expected that the decrease in glucose uptake might not be as great as the decrease in

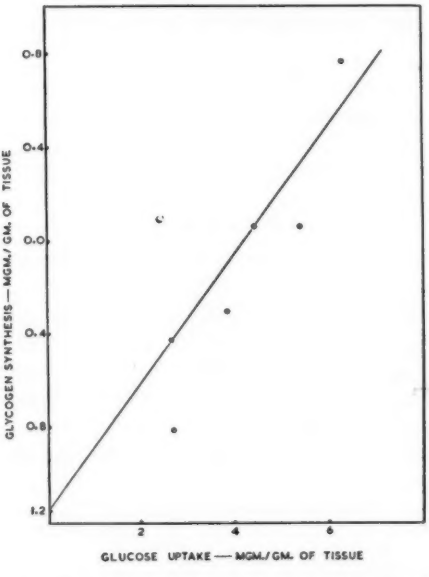

FIG. 3. Glycogen synthesis vs. glucose uptake. Series I.

glycogen synthesis. On the other hand, if each of the reactions leading from glucose to glycogen were inhibited to the same degree, a linear relationship as shown above would still result, but it is felt that this is an unlikely circumstance. The inhibitory effect of potassium, therefore, may lie solely in its effect upon glucose uptake.

One of the objects of the experiment was to determine the influence of potassium upon the insulin effect. The points in Fig. 3, derived from experiments in which potassium, with or without insulin, was used, seem to be in line with the others. This suggests (as shown above) that the insulin effect is neither more nor less susceptible to inhibition by potassium when this ion is present in relatively large amounts than are the usual reactions of glucose uptake or glycogen synthesis, unassisted by insulin.

Summary

1. High concentrations of potassium ion in a buffering medium containing glucose tend to reduce the glucose uptake and the glycogen synthesis in the isolated rat diaphragm.

2. High concentrations of potassium ion in media without glucose do not affect the amounts of glycogen broken down.

3. The inhibitory effect of potassium may lie solely in those reactions concerned with glucose uptake.

4. High concentrations of potassium do not especially affect the "insulin effect" in the isolated rat diaphragm.

5. Diaphragm tissue from fasted rats reacts towards the various conditions studied in qualitatively the same manner as diaphragm tissue from fed rats.

Acknowledgments

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THE EFFECTS OF STRYCHNINE, ESERINE, AND ACETYLCHOLINE ON THE ELECTROCORTICOGRAM AND MOTOR UNIT FIRING; TOGETHER WITH OBSERVATIONS ON THE STRETCH REFLEX IN THE MASSETER MUSCLE OF THE RABBIT[1]

By William D. Wilkey and Frederick R. Miller

Abstract

Observations were made on rabbits and cats under dial anesthesia. Monopolar recording from cortex was used. Strychnine, 1%, on motor cerebral cortex is excitatory, as shown by increased firing of motor units; later the strychnine induces cortical spikes. Each spike is triphasic, consisting of an initial, small positive wave, a large, fast negative wave, and a final, slow positive wave; the first two waves are believed to be excitomotor; the final positive wave is regarded as a positive after-potential with relative quiescence of neurons; it is not excitatory for motor units. Microwaves at high frequency occur during first positive wave and ascent of negative wave; microwaves decay during descent of negative wave and are absent during final positive wave. Microwaves are caused by fast, repetitive firing of neurons; this neuronal firing causes excitation of motor units. Intracortical and extracortical conduction are believed to be repetitive. Acetylcholine (ACh), 1%, on eserinized cortex induces triphasic spikes, resembling those from strychnine; microwaves are likewise present. Strychnine, eserine, and ACh are believed to stimulate cortical synapses. Strychnine and ACh, though very different chemically, are believed to trigger the same fundamental cortical mechanism of conduction.

Introduction

In this investigation the effects on the electrocorticogram and on the motor unit firing were studied, following the application of small amounts of saline solutions of the drugs to the cerebral cortex of the rabbit and cat, anesthetized with dial. The drugs used were strychnine sulphate, eserine sulphate, and acetylcholine chloride (ACh). The method of local application used was introduced by Baglioni and Magnini (5), who applied 1% strychnine, by a small pledget of absorbent cotton or a piece of filter paper, to the motor cerebral cortex of the dog; there resulted characteristic contractions, clearly localized in the contralateral forelimb. The effects were attributed to direct specific action of strychnine on the cortical neurons.

The effects of local strychninization of the cerebral cortex on its electrical activity have been studied by a number of investigators. Bartley (6) described, in the rabbit, the development of large potential strychnine oscillations. Bremer (7), in the 'cerveau isolé' of the cat, observed large 'pulsations strychniques'; when induced in the eyelid motor area, each pulsation evoked contraction of the orbicularis oculi muscle (8).

Dusser de Barenne and McCulloch (17) observed, in the monkey, the large oscillations and designated them 'strychnine spikes', a term now generally

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Contribution from the Department of Neurophysiology, Faculty of Medicine, University of Western Ontario, London, Ontario, Canada. Many of the results in this paper were incorporated in a thesis by W. D. Wilkey; the thesis represented partial fulfillment of the requirements for the degree of Master of Science in the University of Western Ontario. Supported by grants from the National Research Council, Ottawa, Canada.

employed. In further studies in the monkey these workers (18, 27) found that, when recorded by the monopolar method, each strychnine spike is triphasic, being composed of an initial, small positive wave, a large, fast negative wave, and a final, slow positive wave. A microelectrode, inserted to the level of the deep pyramids (Vth layer), shows phase reversal of the constituent waves. Observations with the microelectrode, together with those after thermocoagulation of the superficial layers, led these authors to the following conclusions. Each wave of the spike is negative at the site of its generation and appears as a positive wave a short distance away. Thus the first surface-positive wave is negative where generated, i.e. at the deep pyramids; this wave gives rise to impulses to adjacent cortical areas and also to an ascending disturbance in the superficial layers, yielding the large surface-negative wave (positive below superficial layers); the disturbance then descends to fire the deep pyramids, causing the final surface-positive wave, together with the discharge of efferent impulses. Our own conclusions are in agreement as to the mode of causation of the first two waves but differ with regard to the final surface-positive wave; this we regard as a positive after-potential, with relative quiescence of the neurons.

The stimulating action of 1% eserine on the motor cerebral cortex was determined by Miller (30). Miller, Stavraky, and Woonton (32) recorded 'ACh spikes', induced by the application of 1% ACh to the eserinized cortex; the spikes indicate cortical stimulation, as shown by the accompanying muscular contractions. The demonstration that ACh is capable of stimulating the cerebral cortex is of interest in relation to the suggestion of Dale (14) that autogenous ACh may act as transmitter at certain central synapses; however, the amount of the substance at synapses under physiological conditions would be extremely minute and not comparable with that resulting from the application of a 1% solution to the surface.

This difficulty was met by Miller and Scott, working in the Department of Physiology, University of Toronto. Observations (unpublished) were made on the motor cerebral cortex of the cat under dial. Eserine, 2 mgm., was injected intravenously and caused the characteristic changes in the electrocorticogram; 0.05 ml. of ACh 10^{-6} solution was then injected by a fine needle syringe into the cortex, just below the surface: this induced, in 15 sec., typical ACh spikes of triphasic form, comparable to those from 1% ACh, to be described later in this paper. The specific character of this ACh response was proved by the fact that it was abolished and precluded by intravenous atropine. Thus ACh is capable of stimulating the cortex in a concentration of 1 : 1 million, conceivably in the range of that which may be present normally at synapses.

'Microwaves' during parts of the cortical strychnine spike will be described in this paper; they were first observed in the deeper layers of the cortex of the cat by Wilkey on May 3, 1948. An account of these waves, referred to as 'fine, fast waves', was given by Wilkey (33) and at the same time the record in Fig. 5 was shown. The microwaves were reported also in 1951 and 1953 (34, 35).

Methods

Our observations were made in a large series of rabbits and cats; anesthesia was by dial, injected intraperitoneally in a dose of 0.5 ml./kgm. In the rabbit the electrocorticogram was recorded from the area of mastication described by Ferrier (19); the area on the frontal cortex was localized by preliminary faradization. Motor units were recorded in the *ipsilateral* masseter muscle, this procedure being justified, since the cortical representation of the masseter is bilateral (Miller (29)). In the cat the electrocorticogram was recorded from the anterior sigmoid gyrus, the electromyogram from the contralateral biceps; cortical localization was by preliminary faradization. In a few experiments the electrocorticogram was recorded from the posterior sigmoid gyrus.

The electrocorticogram was recorded by the monopolar method, regarded by Dusser de Barenne as most reliable when drugs are applied to the cortex; in the earlier experiments the stigmatic electrode was a small camel hair brush, secured round the end of a chlorided silver wire; in later experiments the brush was replaced by a silver wire ending in a U loop, the latter being applied to the cortex. The stigmatic electrode was applied to the mastication area in the rabbit or to the anterior (occasionally the posterior) sigmoid gyrus in the cat. The indifferent electrode was a thin silver plate, suitably molded, applied to the parietal area of normal cortex in the rabbit or cat.

For recording from the depths of the cortex and, occasionally, from the surface, a microelectrode was used: this consisted of a small glass tube, drawn off to a tapered tip, enclosing a fine stainless steel wire, 50 to 75 microns in diameter; the indifferent electrode was the silver plate, as already described. All electrodes were supported by upright rods, clamped below to the animal board; rack and pinion arrangements served for placement of the electrodes. The microelectrode was adjusted as to depth by a micrometer, mounted on an upright, secured below to the animal board.

Electromyogram recording was from the 'motor units' by the method of Jasper and Ballem (24): the stigmatic electrode was a fine sewing needle, insulated except at the tip, and supported by a coil of thin copper wire, the latter forming part of the circuit; the indifferent electrode was a silver plate, applied over the exposed muscle at its origin. These electrodes were secured to uprights, clamped below to the animal board.

The head of the animal was secured in a holder clamped to the animal board. The cortex having been exposed, the whole preparation secured to the board, with electrodes in position, was placed in a large, shielding box; this was constructed of layers of silicon steel, interleaved with layers of copper. This type of construction was recommended by Metcalf and Dickinson (28); it is most efficient for excluding outside interference, even in the case of the very high amplifications used in many of our experiments.

Strychnine sulphate and eserine sulphate were dissolved in buffered Locke's solution. In the case of acetylcholine (ACh) sodium bicarbonate was omitted

from the Locke's solution, since ACh is hydrolyzed rapidly in an alkaline medium. Strychnine was used in 1 to 3% solution, as was done by Dusser de Barenne and McCulloch (18). Eserine and ACh were used in 1% solution, as in the work of Miller *et al.* (32).

The various drug solutions were applied to the cortex close to the stigmatic electrode by the method of Baglioni and Magnini; in our earlier experiments this was done by a 4 mm. square of spot test paper, first soaked in the solution, all excess being removed; in later experiments the solution was painted in minimal amount round the electrode by a fine camel hair brush. The method of Baglioni and Magnini, when used with suitable precautions, has definite advantages over that of intravenous injection: in the first place the solution is limited to the site selected and diffusion to the neurons is rapid and effective; also, complicating influences, especially those on the heart, are avoided. The method has been used extensively by Dusser de Barenne and McCulloch in their studies on 'Physiological Neuronography'. Great care was taken to ensure that the effects observed were due to local action of the drug and not to systemic absorption. The occurrence of a striking response after a brief latency (often a few seconds) indicated local action; the duration of this period of drug action was sufficient for taking many records. The development of abnormalities in the electrocorticogram or of generalized convulsions (after a longer application of strychnine or ACh) indicated absorption and, in consequence, the subsequent results were disregarded.

The paired, push–pull amplifiers, designed by Woonton (36), were employed for recording; each amplifier consisted of a preamplifier and three condenser-coupled stages. The amplifiers were operated entirely by batteries, an arrangement particularly suited to recording the microwaves studied in our experiments. The outputs were connected to two cathode-ray tubes, the screens of which were photographed on moving paper. The cortical electrodes were connected to one channel of the amplifier, the muscle electrodes to the other. With the maximal amplification used 100 μv. amounted to 44 mm. on the recording paper.

It happened often, at the beginning of an experiment, that little, if any, activity was shown in the motor units; while this might mean simply a resting condition of the muscle, it was necessary to determine whether or not the placement of the needle electrode was efficacious. For this purpose cutaneous stimulation was applied to the body and, given proper electrode position, activity was induced in the motor units. Sometimes the masseter of the rabbit was stretched by applying the graded tension of a strong coiled spring, attached to the lower incisor teeth; usually one motor unit, or several of these, then began firing. This response constituted a 'stretch reflex' of the masseter. Failing clear responses, reinsertions of the needle were made until a satisfactory placement was secured. Having established the efficacy of the needle electrode, the spring tension was relaxed before application of the drugs to the cortex; under these conditions a weak stretch reflex was present constantly owing to slight tension on the masseter.

Results

Motor Unit Responses

As stated in the Introduction, this paper is concerned with the effects of locally applied drugs on the electrocorticogram, together with the associated responses of the motor units of the muscles. At this point it is necessary briefly to consider the nature of the motor units and their activity. The term 'motor unit' designates a group of muscle fibers, usually about 150 in number, together with the single nerve fiber supplying the group (Clark (12), Creed *et al.* (13)). Landau (25) has emphasized the fact that the muscle fibers, included in a single motor unit, are scattered in three dimensions; this fact has important bearings on the pickup by a needle electrode. We shall refer to the response of a motor unit as a 'motor unit spike' or simply as a 'spike'.

Now, it was shown by Adrian and Bronk (1, 2) that the discharges (spikes) of a motor unit correspond to the repetitive discharges (action potentials) of the single nerve fiber supplying the unit; in other words, a single nerve impulse, if of threshold strength, evokes a single motor unit spike. The following statement applies to normal, unfatigued muscle. The nerve impulse, being transmitted at the neuromuscular ending, induces an action potential (with associated contraction), which proceeds in the muscle fiber as a wave in each direction from the nerve termination. Let us suppose that the needle electrode is at or near the middle of the active muscle fiber: the approach of the wave to the electrode yields a positive deflection, the wave itself a large negative deflection, and its retreat a final positive deflection (25). Thus the motor unit spike is triphasic, the negative deflection being most marked; it is obvious that the electrode samples the wave of potential, the result appearing as the motor unit spike. We emphasize again that a motor unit spike is evoked by a single nerve impulse in the controlling nerve fiber.

Stretch Reflex in the Masseter Muscle of the Rabbit

The effects of drugs on the motor unit responses were studied as an essential part of this research. Now, since marked augmentation of the unit spikes occurs during the stretch reflex, it is necessary to give a short account of this reflex. As stated under 'Methods', downward traction applied by the coiled spring to the mandible of the rabbit increases greatly the firing of units of masseter: the effects are shown in Fig. 1; they were recorded in an anesthetized animal but, since similar effects may be induced in the decerebrate condition, the reactions in Fig. 1 may be regarded as illustrating a bulbar 'stretch reflex'. It is comparable to the spinal stretch reflex in the vasto-crureus muscle, first described by Liddell and Sherrington (26). Motor unit responses in the soleus muscle during the stretch reflex were recorded by Denny-Brown (15).

Fig. 1 may be interpreted as follows. In *A* there is slight traction on the jaw, resulting in infrequent impulses from a few muscle spindles, impinging on motoneurons of the motor part of the Vth nucleus; this connection would be devoid of internuncials. In the weak stretch reflex of *A* firing is recorded of a

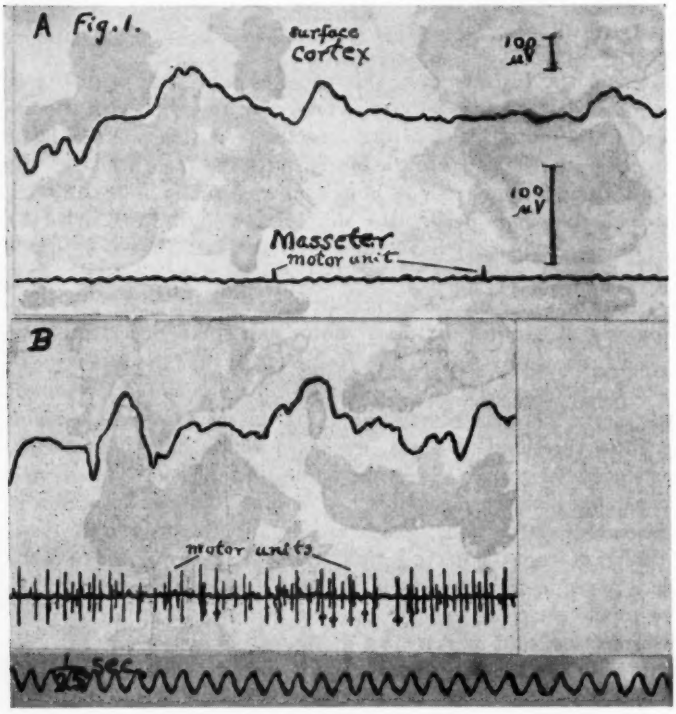

FIG. 1. Rabbit. Stretch reflex in masseter muscle. *A*, *B*, upper records: electrocorticogram of cortical mastication area; lower records: motor units of ipsilateral masseter. Cortical innervation of masseters bilateral. *A*, before stretch of masseter. *B*, after stretch of masseter. Explanation in text. Time in all records 1/25 sec.

single unit, which represents the firing of a controlling single Vth motoneuron. More powerful traction on the jaw yields the effects shown in *B*; it may be supposed that the original unit of *A* continues firing in *B*, as shown by the smaller series of spikes; clearly this firing is much increased in frequency and positive deflections appear. There is further recruitment of additional units, with positive deflections, as shown by the larger spikes. In *B*, as compared to *A*, it may be supposed that the Vth motoneurons are bombarded by impulses from more spindles, responding at faster rates; there result faster firing of the motoneurons and recruitment of more of these, also firing at high frequency. The motoneuron activities are signalized by the spikes in the muscle.

Early Effects of Application of Strychnine to Motor Cerebral Cortex

That locally applied strychnine exerts a stimulating effect on the cerebral cortex before the appearance of cortical strychnine spikes is shown by the electromyogram of the masseter muscle of the rabbit: the rate of the motor unit spikes is increased and there is recruitment of additional units; these

early effects are shown in Fig. 2. Before application of strychnine only occasional firing of a motor unit occurred (prior to *A* of Fig. 2). The first application of 0.1% strychnine induced the motor firing in *A*; a second application of the same concentration caused a slight but perceptible increase

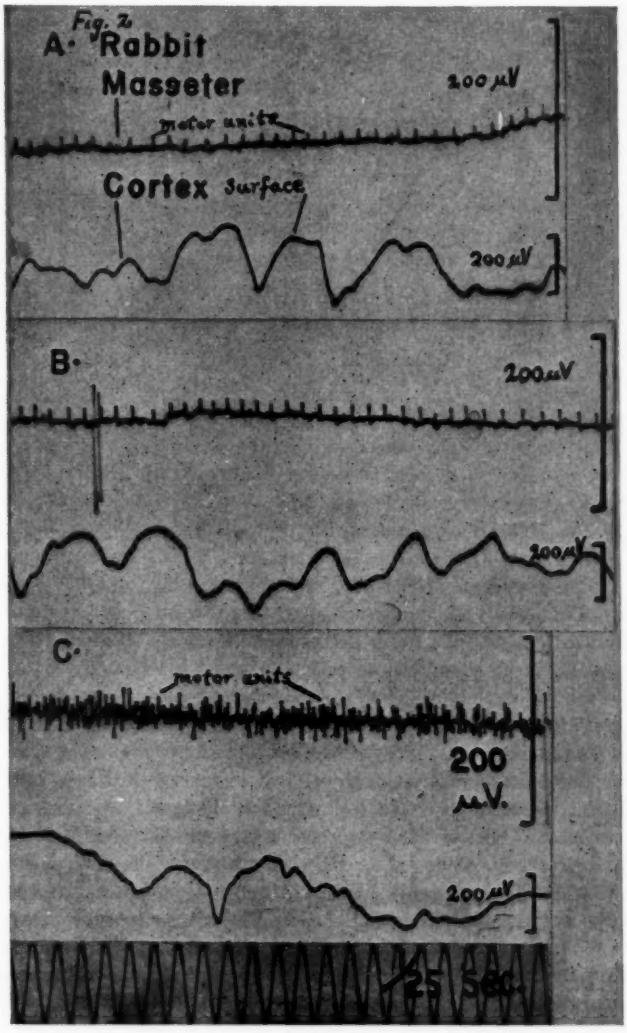

FIG. 2. Rabbit. Early effects of strychnine on cortex. *A*, *B*, *C*, upper records: motor units of masseter; lower records: electrocorticogram of cortical mastication area. *A*, 30 min. after 0.1% strychnine on cortex. *B*, after further application of 0.1% strychnine to cortex. *C*, after 1% strychnine on cortex. Explanation in text.

in the rate of the spikes in *B*. Then 1% strychnine caused the marked increase in rate of the spikes in *C*, together with recruitment of many more units.

The electrocorticogram showed progressive changes: the second application of 0.1% strychnine caused reduction in amplitude of the large waves, with increase in number of the small waves (*B*); in *C*, after 1% strychnine, the large waves almost disappeared, while the small waves were more numerous; there was one abortive spike. These effects are proved, by the accompanying increased motor unit firing, to represent cortical excitation by strychnine; they may be ascribed to intense, asynchronous activity of the cortical neurons; this explanation is similar to that originally given by Bartley (6). This intensified cortical activity in turn induced increased activity of the Vth motoneurons, with increased rate of firing and the recruitment of more neurons; these effects manifested themselves in the enhanced motor unit firing described above. The apparent increase in the action potentials of the motor units in *C* may be ascribed to recruitment of more units, the action potentials of which were added to those of the units already active because of synchronous firing.

Cortical Strychnine Spikes

In approximately 60 sec. after the cortical application of 1% strychnine typical spikes appear; they are shown in the rabbit's cortex in Fig. 3; the spikes occur first singly, then in bursts of three or four, and finally as a train of uniform amplitude and wave form. Each spike is triphasic, consisting of a small positive, a large negative, and a final small positive wave. As stated in the 'Introduction' Dusser de Barenne and McCulloch observed, in the monkey, a phase reversal of the waves of the spike at the level of the deeper pyramids. This result we confirmed in the rabbit and cat: for this purpose we used the microelectrode described under 'Methods'; when this electrode was inserted into the mastication area of the rabbit's cortex to a depth of 0.75 mm. (approximately the level of the deeper pyramids), reversal of the waves began and became complete at 1 to 1.5 mm.; the result at this latter depth is shown in Fig. 4. Thus, while the sequence of waves at the surface is positive, negative, and positive, at depth it is negative, positive, and negative.

Our observations lead us to the following conclusions. Activity of the deep pyramids causes, at this level, a negative wave, which appears at the surface as the first positive wave. The depth-negative wave sends impulses to muscles and also to the superficial layers; the wave is thus excitomotor. The resulting activity in the superficial layers yields the large surface-negative wave, which appears as a positivity below these layers. These conclusions are based on those of Dusser de Barenne and McCulloch. However, we do not concur in the opinion of these authors that the final surface-positive wave is due to return of activity from superficial layers to deep pyramids. On the contrary, our results, to be described later, imply that the final surface-positive wave represents, in reality, a positive after-potential, with relative neuronal quiescence.

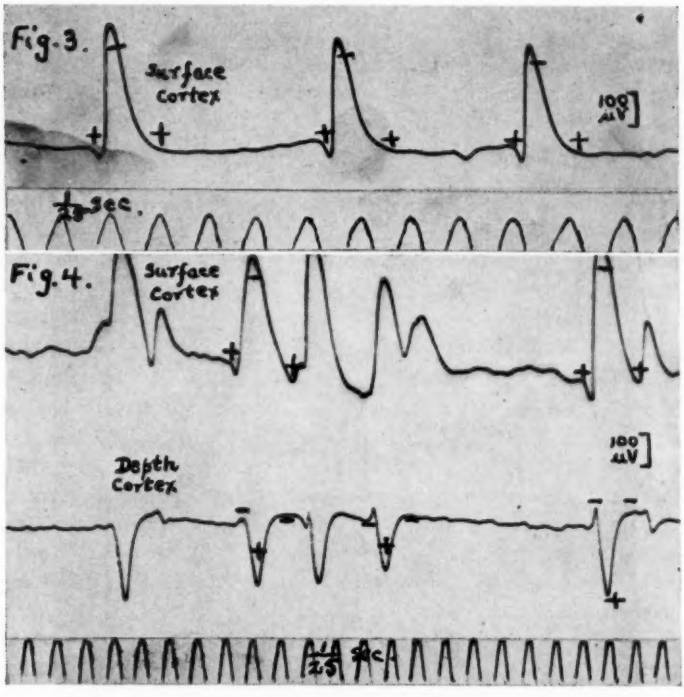

FIG. 3. Rabbit. Train of triphasic strychnine spikes after 1% strychnine on cortical mastication area. Explanation in text.

FIG. 4. Rabbit. Reversal of strychnine spikes in depth of cortex. After 3% strychnine on mastication area. Upper record: surface of cortex; lower record: 1.5 mm. depth with microelectrode. Explanation in text.

Microwaves During the Cortical Strychnine Spike

An account will now be given of remarkable, fast microwaves, which are revealed within the cortex during certain parts of the strychnine spike. They are shown in Fig. 5, taken from an experiment by Wilkey on May 3, 1948. Surface and depth records were obtained from the anterior sigmoid gyrus of a cat under dial; 3% strychine had been applied to the surface of the gyrus. Surface record was obtained, at low amplification, by a loop of silver wire; depth record by a microelectrode, inserted through center of loop to level of deep pyramids; microelectrode was of type described under 'Methods'. A silver plate on parietal cortex served as the indifferent electrode for surface and depth circuits. In the depth circuit a 0.01 μf. condenser was inserted just beyond microelectrode and a similar condenser beyond indifferent plate; condensers served as filters to reduce size of large waves and thus reveal microwaves. Amplification of depth record was very high, as shown by calibration on record.

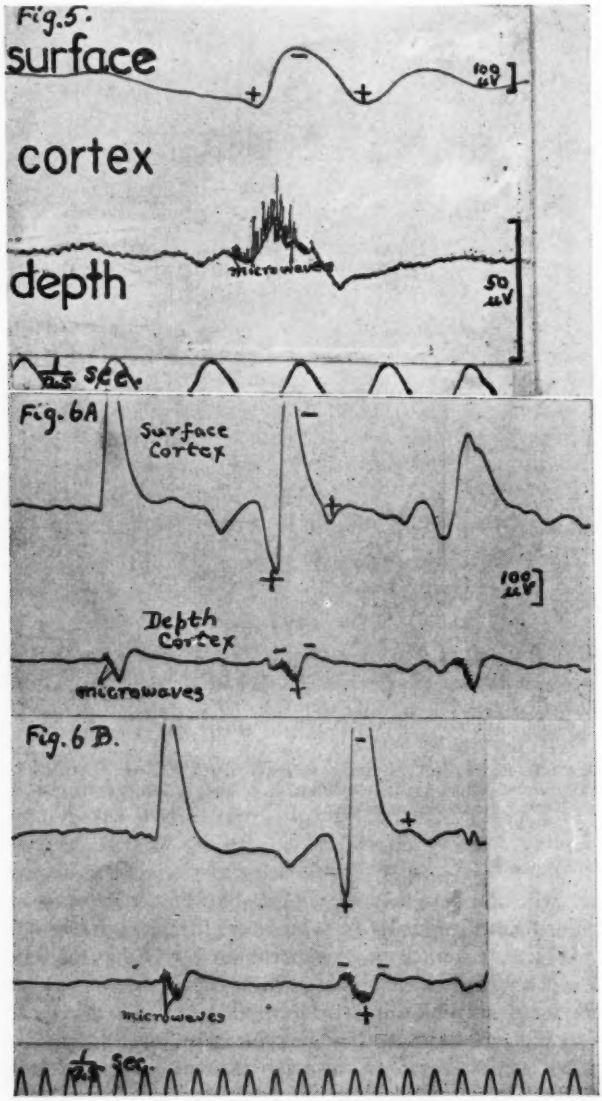

FIG. 5. Cat. Microwaves in depth of cortex; after 3% strychnine. Upper record: surface of anterior sigmoid gyrus; low amplification; lower record: 1.5 mm. depth with microelectrode; very high amplification; condensers; microelectrode. Depth record shifted slightly to left because of condensers.

FIG. 6. Cat. Anterior sigmoid gyrus after 3% strychnine. Microwaves at depth; low amplification. *A*, *B*, upper records: surface of cortex; low amplification; lower records: microelectrode at 1.5 mm. depth; low amplification; no condensers; microwaves shown; correspond to first surface-positive wave and ascent of surface-negative wave. Explanation in text.

Fig. 5 shows that the microwaves occur during first surface-positive wave and ascent of surface-negative wave; they decay gradually during descent of surface-negative wave and are absent during final surface-positive wave. The rate of the microwaves cannot be exactly determined at this paper speed but other records suggest that it is slightly higher than 1000/sec.; this rate would be consistent with a refractory period of 0.5 msec. for the neuron. Probably the most significant fact is that this rate is within the range of that observed by Adrian and Moruzzi (4) of impulses in the pyramidal tract during the cortical strychnine spike. Anticipating our later results, we may say at once that the microwaves represent repetitive firing of the deep pyramids and that this firing is the cause of the pyramidal tract impulses of Adrian and Moruzzi.

While recognizing the cogency of the inference just drawn it is essential to establish, beyond all doubt, the genuine physiological nature of the microwaves; evidence in support of this thesis will be presented systematically in the following sections. In the first place the possibility must be considered that the microwaves might represent injury discharges induced by the insertion of the microelectrode. Injury discharges resulting from cuts or punctures of the cortex of the cat were fully studied by Adrian and Matthews (3). However, the microwaves in our experiments cannot be regarded as injury discharges for the following reasons. First of all, they occur only at definite times in the strychnine spike, specifically during the first surface-positive wave, the ascent of the surface-negative wave, and the beginning of the descent of that wave, when they gradually decay. They do not occur between the spikes, as would be expected were they injury discharges. Further, the frequency of the microwaves is in the range of 650 to 1000/sec., which is much higher than the rate of 30 to 76/sec. found by Adrian and Matthews for the injury discharges in the cortex of the cat. An important point, to be referred to later, is that the microwaves may be picked up by the microelectrode, when applied to the *surface* of the cortex, without any injury whatever (Fig. 8*A*) and these surface microwaves correspond closely to those at depth. The voltage of the microwaves varies from 5 to 20 μv.; these voltages are clearly very much lower than the voltage of the injury discharges shown in Fig. 6 of the paper by Adrian and Matthews, although the voltage in that figure is not indicated. The above considerations establish definitely that the microwaves in our experiments are not injury discharges.

The possibility that noise background of the tubes might contribute to the microwaves was also considered; to settle this question electrodes, arranged as in the actual experiments, were inserted into the brain of the dead cat; at the amplification of the experiment shown in Fig. 5 moderate noise background was apparent, but it differed completely in appearance from the microwaves. However, for added certainty, a number of experiments were carried out by Miller at lower amplifications, at which controls in the dead brain gave assurance of complete absence of noise background; in several instances condensers

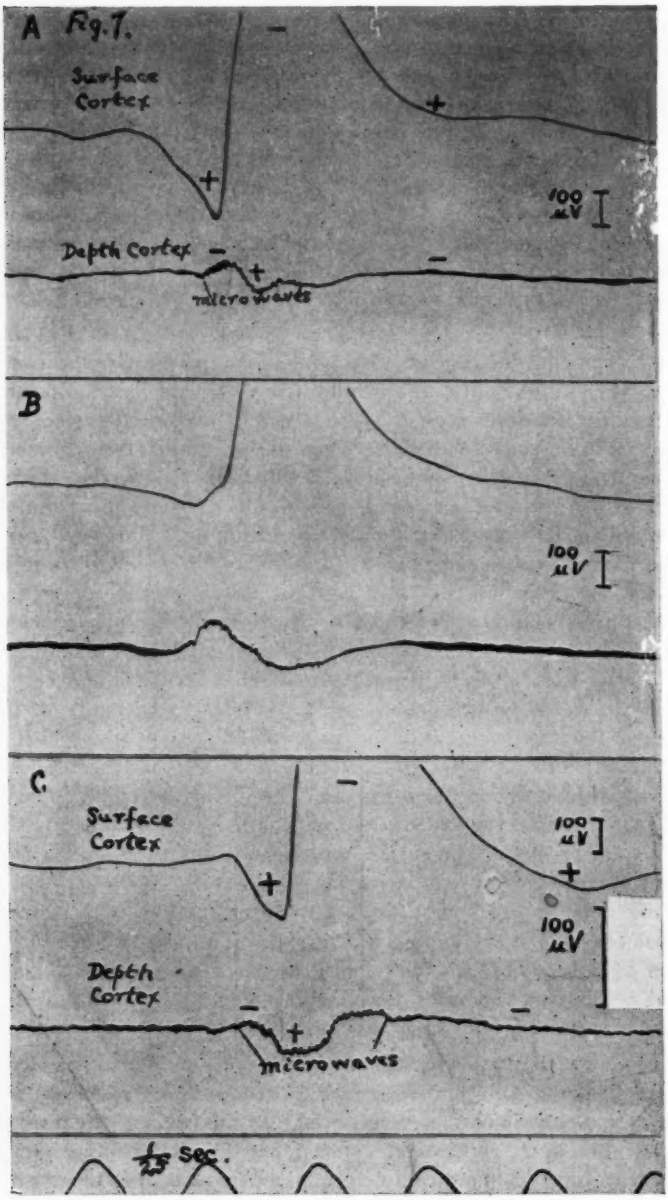

FIG. 7. Cat; continuation of Fig. 6; faster recording speed. *A*, *B*, same amplification as in Fig. 6. *C*, upper record: low amplification as in *A* and *B*; lower record: high amplification; condensers present; microwaves pronounced. Explanation in text.

were omitted from the microelectrode circuit. In all experiments, including those without condensers, characteristic microwaves were recorded. Details of these experiments will now be given.

In Fig. 6, *A* and *B*, the microwaves are shown at 1.5 mm. depth at a comparatively low amplification and ***without condensers*** in the microelectrode circuit; depth record shows phase reversal of larger waves. Microwaves occur during first surface-positive (depth-negative) wave and also during ascent of surface-negative (descent of depth-positive) wave. Similar results are shown later in the same experiment at faster recording in Fig. 7, *A* and *B*, also without condensers; these figures also show decay of microwaves in descent of surface-negative wave. Thus all the essential features of the microwaves are shown without condensers and at comparatively low amplification. In Fig. 7 *C*, also from the same experiment, at higher amplification and *with condensers* in depth circuit, similar results are shown: the microwaves, more highly amplified, correspond to the surface-positive wave and the ascent of the surface-negative wave; they continue, with gradual decay, into the beginning of descent of surface-negative wave.

In Fig. 8 *A* a strychnine spike was recorded *at the surface* of anterior sigmoid gyrus of cat *with a microelectrode* at the high amplification usually employed for depth. For wave identification another surface record was taken at lower amplification with a silver loop electrode, embracing the point of application of the microelectrode. The microwaves shown by the microelectrode at the surface correspond (as at depth) to the first surface-positive wave, the ascent of the surface-negative wave, followed by their decay in the descent of the same wave. Microwaves are absent during the final surface-positive wave, which is of smooth contour. For comparison the microelectrode was then inserted to a depth of 0.75 mm. (Fig. 8 *B*) and it is seen that the depth microwaves are quite comparable to those at the surface (Fig. 8 *A*); they are also absent during the final surface-positive wave. Thus it may be concluded that the microwaves at surface and depth represent similar phenomena, namely fast, repetitive firing of cortical neurons at various levels.

The microwaves during parts of the strychnine spike, reported thus far, were recorded with a microelectrode by the monopolar method. Additional evidence for the genuineness of these waves, as products of neuronal activity, might be expected by the use of bipolar electrodes, although these would not reveal the familiar form of the gross strychnine spike, as recorded by the monopolar method. For this purpose a pair of fine steel needles, insulated except at their tips and 1.25 mm. apart, were inserted to a depth of 0.8 mm. (approximately the level of the deep pyramids) into the posterior sigmoid gyrus of the cat; the usual two condensers were included in the circuit. Control surface recording was by a loop of silver wire, embracing the site of insertion of the bipolar microelectrodes. The recordings shown in Fig. 9 were obtained after application of 1% strychnine to the posterior sigmoid gyrus. For the reasons given above, the gross pattern of the bipolar record does not duplicate exactly that of the monopolar record. However, the essential

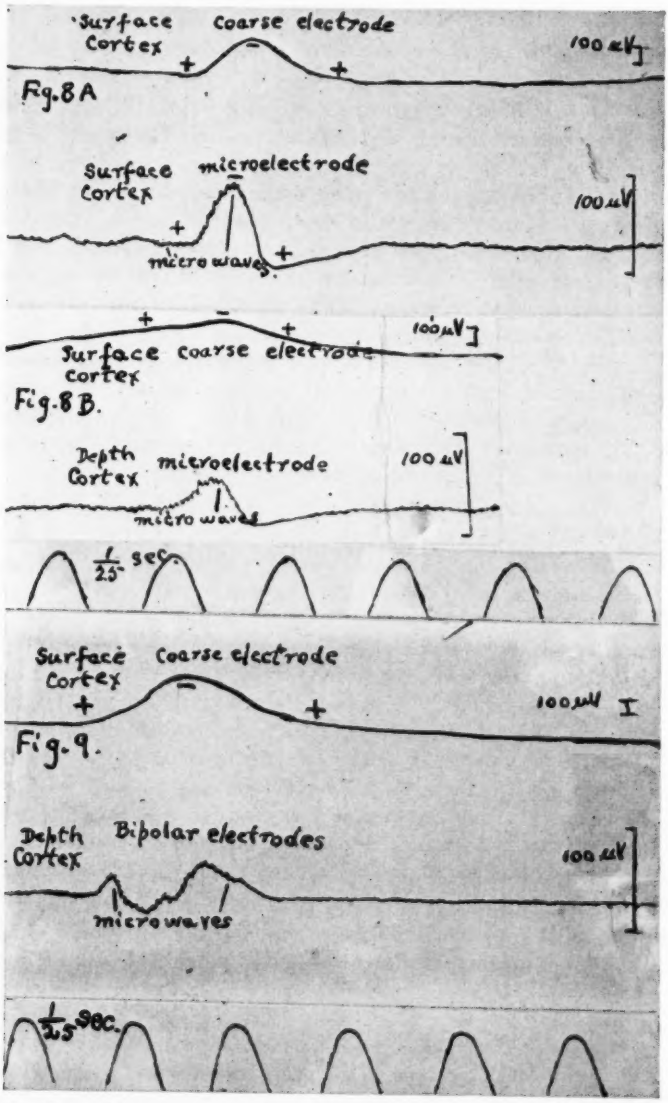

FIG. 8. Cat. Microwaves at surface of anterior sigmoid gyrus after 3% strychnine. *A*, upper record: surface of cortex; coarse electrode; low amplification; lower record: surface of cortex by *microelectrode;* high amplification; condensers: microwaves shown. *B*, upper record: surface of cortex; coarse electrode; low amplification; lower record: microelectrode at 0.75 mm. depth; high amplification; condensers: microwaves shown. Explanation in text.

FIG. 9. Cat. Posterior sigmoid gyrus after 1% strychnine. Microwaves at depth by *bipolar* needle electrodes. Upper record: surface; coarse electrode; low amplification; lower record: bipolar electrodes at 0.8 mm. depth; high amplification; condensers: microwaves shown. Explanation in text.

fact is that microwaves occur during the course of the surface strychnine spike and these microwaves (Fig. 9) correspond in a general way to the first surface-positive wave, as well as to the ascent and beginning of the descent of the surface-negative wave, as determined repeatedly by the monopolar method. Thus typical microwaves can be recorded by the bipolar method, which is strictly localizing in respect to derivation. In conclusion we may state that microwaves owe their origin to potential fields developed by the repetitive firing of deep and superficial neurons.

We shall now consider more particularly the microwaves generated by repetitive firing of the deep pyramids; this firing is clearly related to the studies of Adrian and Moruzzi (4) on the pyramidal tract of the cat; they observed, during the cortical strychnine spike, impulses at 500 to 1000/sec. in the tract. Some of these impulses they attributed to single fibers, others to several fibers, originating in a group of synchronized cortical neurons. It is obvious that the frequencies of the strychnine microwaves from the deep pyramids, of the order of 1000/sec. or less, correspond closely to those from the pyramidal tract. Hence we may conclude that repetitive firing of the deep pyramids (represented by microwaves) is the cause of the repetitive impulses in the pyramidal tract.

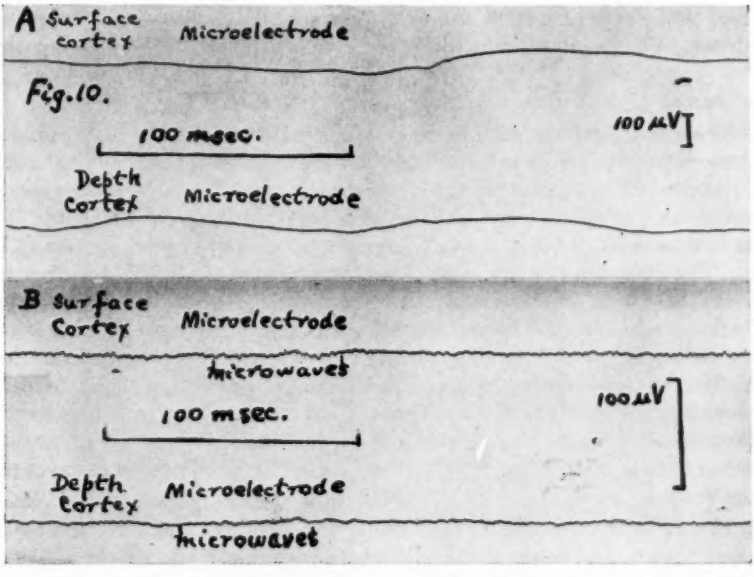

FIG. 10. Cat. Posterior sigmoid gyrus. Microwaves shown without strychnine. *A*, *B*, upper records: microelectrode on surface; lower records: microelectrode at 0.9 mm. depth; two circuits independent. *A*, low amplification; no condensers; usual waves at surface and depth. *B*, high amplification; condensers in each circuit : microwaves shown at surface and depth. Explanation in text.

Cortical Microwaves Without the Application of Strychnine

The question now arises whether microwaves (indicating repetitive firing of neurons) occur in the cortex without the application of strychnine. The subject was investigated by Miller and his records are shown in Fig. 10. The tip of a microelectrode was applied to the surface of the posterior sigmoid gyrus in a cat; a similar microelectrode was inserted, close to the first, to the level of the deep pyramids. The indifferent electrode for the surface and depth record was, in each case, a silver wire, ending in a U loop, the latter applied to the surface so as to embrace each microelectrode; the openings of the two U loops faced each other; thus surface and depth circuits were independent, each being connected to a channel of the amplifier.

Fig. 10 *A* shows a surface and depth record at low amplification, the usual large and small waves being shown in both records. In Fig. 10 *B* high amplification was used and two condensers were inserted in each circuit to filter out the larger waves. The surface record shows microwaves at 700/sec., while the depth record shows them at 450/sec. The possibility of the microwaves being due to noise background was excluded by controls in the dead brain, which showed no such waves. The microwaves thus indicate repetitive neuronal firing and it is apparent that this is a fundamental physiological activity, which strychnine is capable of intensifying. Hence we may conclude that normally the somata fire repetitively; further that conduction, intracortical and extracortical, is normally by repetitive impulses in axons and dendrites, in accordance with Adrian's principles.

Relationship of Microwaves to Motor Units

Before considering the relationship of the microwaves to the motor units of the muscle it must be stated that the strychnine spike, regarded as an entity, and recorded at low amplification (not revealing the microwaves), represents a convulsive cortical activity, which evokes gross contraction in muscle. The spike is thus comparable to the discharge of impulses in a strychninized spinal frog. The effects of the spikes on the jaw muscles of the rabbit are shown in Fig. 11; here the jaw movements were recorded by a small, variable saline potentiometer, connected to one channel of the amplifier; the mastication area of the cortex was connected to the other channel. Each spike, if of sufficient amplitude, evokes depression of the jaw (contraction of digastrics), followed by elevation (contraction of masseters). This result, which is similar to that of Bremer on the orbicularis oculi, shows that the spike emits excitomotor impulses to muscles. We may now relate these effects to the microwaves and motor units.

In Fig. 12 a strychnine spike is shown at the surface of the anterior sigmoid gyrus of the cat and below it the motor unit response of contralateral biceps. At the slow speed of *A* the unit response corresponds to the first surface-positive wave and extends into the time of ascent of the surface-negative wave. Effects are more clearly shown at the faster speed of *B*; here one spike of the unit response corresponds to the surface-positive wave and may be attributed

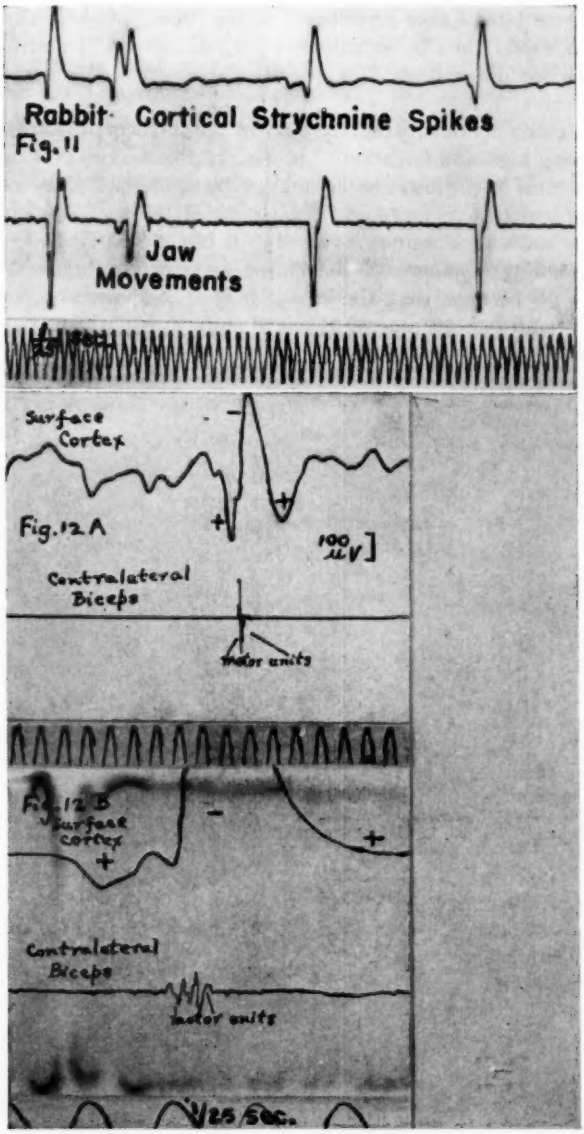

FIG. 11. Rabbit. Strychnine spikes after 1% strychnine on mastication area. Spike evokes jaw movement. Upper record: surface of cortex; lower record: jaw movements recorded by variable saline potentiometer. Explanation in text.

FIG. 12. Cat. Anterior sigmoid gyrus after 1% strychnine. Strychnine spike evokes firing of motor unit in contralateral biceps. *A*, upper record, surface of cortex; lower record: motor units in contralateral biceps. *B*, *idem* at faster recording speed. Explanation in text.

to it; the three later spikes correspond to the ascent of the surface-negative wave, which would thus be excitomotor, in addition to the surface-positive wave. Here we differ from McCulloch, who regards the first wave only as excitomotor.

These conclusions are substantiated when the intracortical microwaves are recorded, using high amplification. In Fig. 13 the microwaves are shown at a depth of 1 mm. in the anterior sigmoid gyrus of the cat: they occur during the first surface-positive wave and the ascent of the surface-negative wave; the gross cortical waves are not reversed in *A* but are partially reversed in *B*. The corresponding responses of the motor units of contralateral biceps are shown. It will be seen that the motor unit spikes correspond to surface-positive wave and to beginning of ascent of surface-negative wave; a tetanus

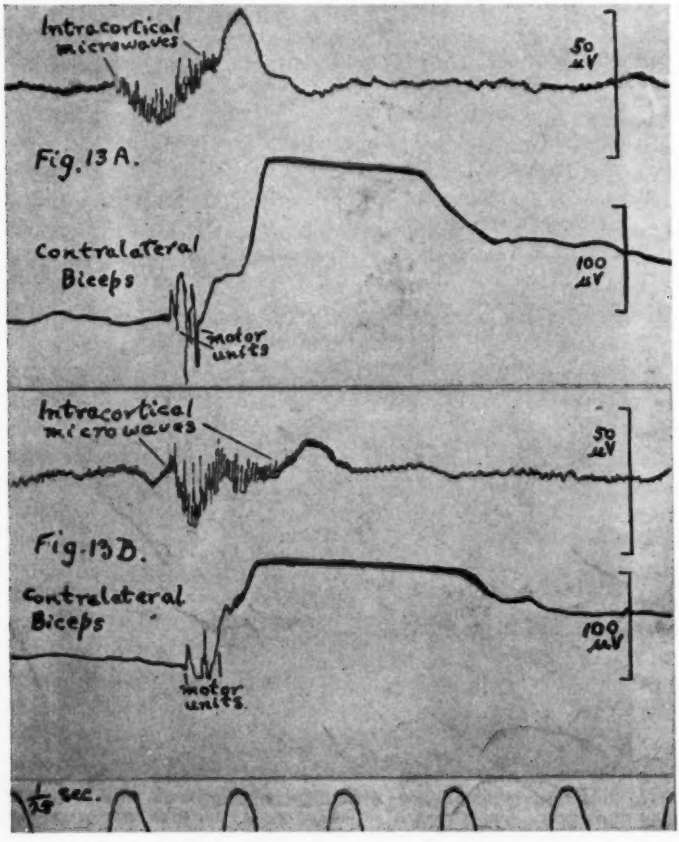

FIG. 13. Cat. After 1% strychnine on anterior sigmoid gyrus. Intracortical microwaves induce motor unit firing. *A*, upper record: microwaves by microelectrode at 1 mm. depth; high amplification; condensers; lower record: motor unit firing in contralateral biceps. *B*, continuation of *A*: effects similar. Explanation in text.

of biceps then follows and corresponds to later part of ascent of surface-negative wave; this tetanus was detectable by palpation of muscle. From the records in Fig. 13 and other records we infer that the ascent of the surface-negative wave, with its microwaves, is excitomotor, in addition to the surface-positive wave.

As in the cat, microwaves were shown to occur within the cortex of the rabbit during the strychnine spike, their rate of 680/sec. being within the range of those in the cat. The strychnine spike in Fig. 14 was obtained from the surface of the masticatory area of the rabbit at low amplification and the microwaves are not shown; however, the effects of the microwaves are shown by the burst of repetitive firing in a motor unit of masseter; the burst corresponds in time to first surface-postive wave and ascent of surface-negative wave, a result which supports the conclusion from the cat that both these waves are excitomotor to muscle.

It will be appreciated that the repetitive firing of the cortical neurons operates through motoneurons of the spinal cord for biceps of cat (Fig. 13); also through motoneurons of motor part of Vth nucleus for masseter of rabbit (Fig. 14). The spinal and bulbar motoneurons in each case would fire repetitively in a manner simulating that of the cortical neurons and, presumably, at about the same rate as that of the latter. The resulting rate of firing of the motor unit in Figs. 13 and 14 is naturally lower than that of the controlling neurons, because of the longer refractory period of the muscle fiber (2 msec.) in comparison with that of the neuron (0.5 msec.).

Effects of Eserine and Acetylcholine on the Electrocorticogram and Motor Units

The effects of the application of eserine and ACh to the masticatory area of the rabbit's cortex are shown in Fig. 15: eserine, 1%, decreased the amplitude of the large cortical waves and increased the number of the small, fast waves (*B*); the latency from the moment of application was brief, *ca.* 10 sec., indicating that the effects were due to local cortical action of the eserine. Proof that the cortex was stimulated by eserine is afforded by the electromyogram of masseter (*B*), which showed increased frequency of the spikes, together with recruitment of additional units. These effects of eserine resemble those in the early stage of strychnine action; they may be interpreted in a similar way, as due to fast, asynchronous firing of cortical neurons; as already stated under strychnine, the motor unit effects are brought about through mediation of the Vth motoneurons in the motor part of this nucleus.

In about two minutes after manifestation of the eserine action 1% ACh was applied to the cortex: first there was some intensification of the above eserine action; then large, fast excursions of about 250 μv. developed, at first singly, then in groups, and finally in continuous trains of large, regular excursions of about 400 μv. at a rate of 8/sec., as shown in *C*. Miller *et al.* (32) referred originally to these excursions as 'Es.-ACh spikes', but, since ACh is clearly the trigger for their production, we now prefer to designate them simply as 'ACh

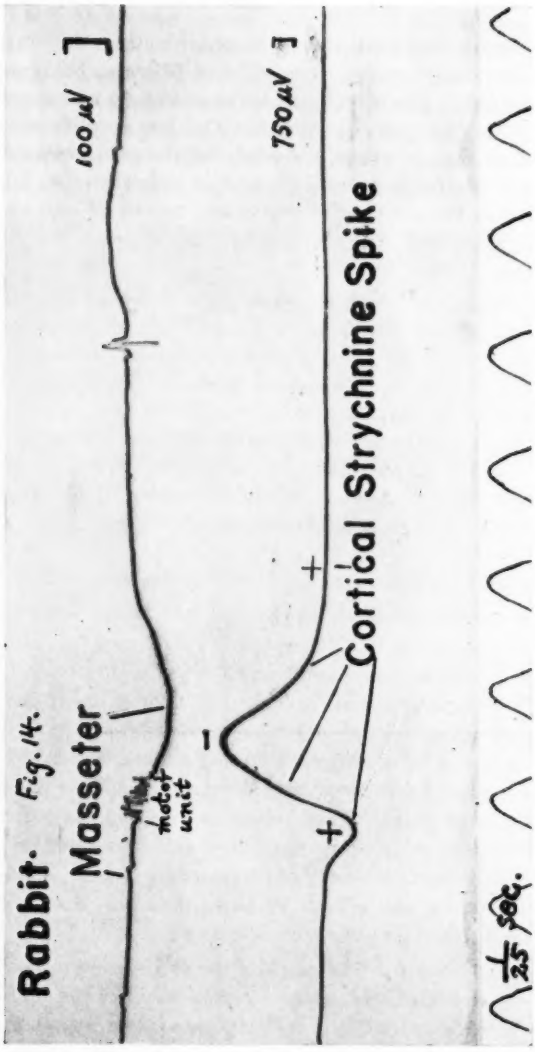

FIG. 14. Rabbit. After 1% strychnine on mastication area. Cortical strychnine spike induces motor unit firing in masseter. Upper record: unit firing of masseter induced by first surface-positive and ascent of surface-negative wave of cortical strychnine spike; lower record cortical strychnine spike; coarse electrode; low amplification. Explanation in text.

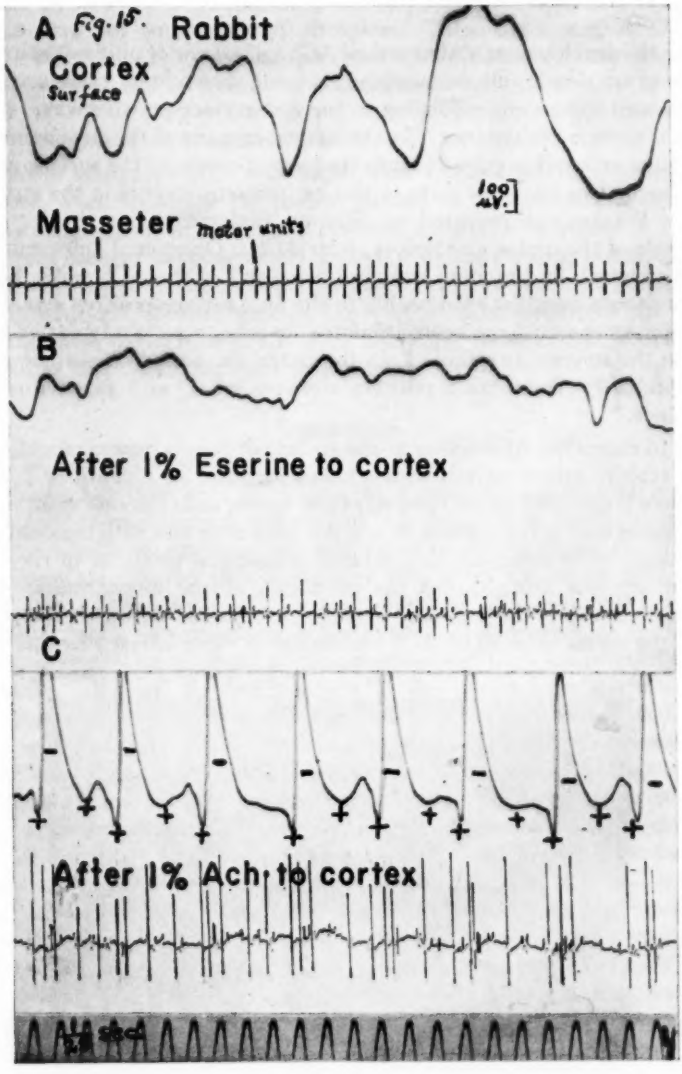

FIG. 15. Rabbit. After eserine and ACh on cortical mastication area; effects on motor unit firing. *A*, *B*, *C*, upper records: cortex, lower records: motor units of masseter. *A*, before drugs; *B*, after 1% eserine on cortex: flattening of cortical waves; increased firing in units of masseter. *C*, after 1% ACh on cortex: triphasic ACh spikes induce characteristic firing in motor units of masseter. Explanation in text.

spikes'. In appearance they resemble closely strychnine spikes: thus each spike is triphasic, consisting of a first positive, a large negative, and a final positive wave.

With the development of the cortical ACh spikes, motor unit spikes increased greatly in amplitude and assumed the grouping shown in *C*: there was a burst of large unit spikes, corresponding to the first surface-positive wave, showing that this wave is excitomotor, like the analogous wave of the strychnine spike; these large unit spikes extended into the time of ascent of the surface-negative wave, suggesting that this wave is also excitomotor, as it is in the strychnine spike. We are not prepared to offer an explanation for the increase in amplitude of the motor unit spikes under ACh. Occasional large motor unit spikes occurred, in a random way, in other parts of the ACh spike, but they did not show a constant relationship to the final surface-positive wave; hence the latter wave would not be excitomotor, an inference already drawn for this wave in the strychnine spike. Like the latter, the final ACh surface-positive wave probably represents a positive after-potential, with relative neuronal quiescence.

Fig. 16 shows the ACh spikes at the surface of the cortical mastication area in the rabbit; also a record, with a microelectrode, at a depth of 1.4 mm.; here there is complete phase reversal of the waves, as in the case of strychnine. Microwaves during the cortical ACh spike were observed with the oscilloscope by Wilkey; they represent fast, repetitive neuronal firing, as in the case of strychnine; this firing causes the excitation of the motor units. These observations lead to the inference that the processes of excitation and conduction in the cortex induced by ACh are similar to those induced by strychnine.

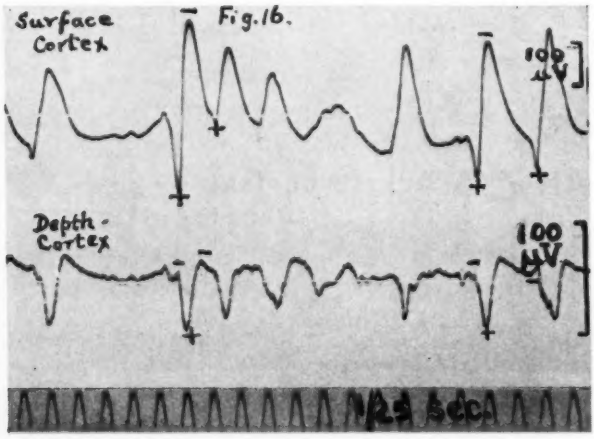

FIG. 16. Rabbit. After 1% eserine and 1% ACh on cortical mastication area. Reversal of ACh spikes at depth. Upper record: ACh spikes at surface of cortex; lower record: microelectrode at 1.4 mm. depth; no condensers. Reversal of spikes. Explanation in text.

However, it must be stated that the effects of ACh are abolished and precluded by intravenous atropine (32); on the other hand the effects of strychnine are not suppressed by atropine. These facts suggest that the basic chemical changes at the synapses would be different for strychnine and ACh; the subject will be considered under Discussion.

Control Observations on Drug Solutions

In control observations it was found that Locke's solution, buffered or unbuffered, when applied to the cortex, yielded no perceptible effects on the electrocorticogram or motor units. Further, a suitable quantity of Locke's solution was brought to the same pH as that of each of the drug solutions used; application of these control solutions to the cortex, in the same amounts as the drug solutions, yielded none of the effects characteristic of the latter. Hence the observed effects of the drug solutions, as described above, were proved to be specific for each of the drugs.

Discussion

Mode of Action of Strychnine

Strychnine has long been recognized as a powerful excitant of the gray matter of the central nervous system and it is desirable to decide whether its primary action is on the neuronal somata or on the synapses or on both. Heinbecker (23) inferred that strychnine stimulates the nerve cells in the ganglionated cardiac nerve of Limulus; however, the possibility exists of action on synaptic-like structures in the ganglion. Moreover, as Bremer (9) remarks, it is unwise to apply conclusions derived from an invertebrate to vertebrates. Opposed to direct action of the alkaloid on somata is the observation of Dusser de Barenne (16) that strychnine, when applied to the cells of spinal ganglia in the dog, has no appreciable effect on reflexes; this lack of effect may be attributed plausibly to the absence of typical synapses in the ganglia. Thus we are induced to return to an earlier view that the primary action of strychnine is exerted on the synapses; apparently this view was tacitly accepted by Dusser de Barenne, who held that the alkaloid acts only where synapses are present, as in the cortex (cited by McCulloch (27)).

From observations on strychnine tetanus in the spinal cord Brooks and Fuortes (11) maintain that the primary action of the alkaloid is a depolarization of the neuron, presumably the soma, and that the depolarization causes efferent discharges. We realize that a distinction between soma and pericellular synapses is highly artificial; however, if depolarization is actually the primary action, we should prefer to place its incidence in the synapses. But, if depolarization should prove not to be the cause but rather the effect of neuronal activity one might speculate as to the possibility that the alkaloid combines with receptors in the synaptic protoplasm. But, whatever the intimate nature of strychnine action may be, on the evidence available, we should place its initial locus in the synapses; its action there would lead to repetitive firing of the neurons. In order to accord with various experimental

results, it is necessary to suppose that strychnine also enhances conductivity at synapses or, as more usually stated, diminishes resistance at these junctions.

It will be clear that the above conceptions of synaptic action provide logical explanations for classical experiments like those on the strychninized spinal frog, showing reduction of reflex latency, increased responses, and generalized convulsions. It may be noted that there is a biological resemblance between the convulsive discharge in a strychninized spinal frog and the cortical strychnine spike, the latter representing a convulsive activity in the cortex with motor discharges.

It has sometimes been asserted that strychnine acts in virtue of being an anticholinesterase substance. In this connection Miller observed that 1% strychnine, applied to the hypoglossal nucleus, excites the motoneurons, as shown by powerful tongue contractions. Now this action is not suppressed by intravenous atropine; hence strychnine is not an anticholinesterase and excitation by it cannot be attributed to the preservation of autogenous ACh.

We have stated several times that the microwaves during the strychnine spike are produced by fast repetitive firing of neurons in the deep and superficial layers of the cortex. Their relationship to a large wave of the spike is most readily discernible at moderate amplification in the course of the large negative wave (Figs. 6, 7, and 8); in this case the microwaves are produced in the superficial neurons. We may now consider the mode of genesis of a single microwave. Adrian and Matthews (3), in their analysis of the ordinary waves of the cortex (without strychnine), conclude that each 'brief wave' is due to synchronous pulsation in a small group of neurons. It appears plausible to infer that each microwave under strychnine is likewise the result of synchronous pulsation or firing in a small neuronal group.

The possible relationship of the microwaves to the larger waves of the strychnine spike will now be examined. Our records show that the microwaves occur during the first positive wave, the ascent of the negative wave, and the beginning of the descent of the latter wave; they then decay and are absent during the remainder of the descent, as also during the final positive wave. These time relationships have been regularly maintained in our records. Now it is generally held that when two phenomena (microwaves and large waves) occur regularly together there must be a causal relationship between them. Here the occurrence of microwaves on the ascent of the negative wave and their decay during the descent may be interpreted as indicating that the microwaves are the cause of the larger wave. It has been suggested occasionally that the large negative wave signalizes a depolarization of certain neurons by strychnine and that this process induces secondarily the repetitive firing (microwaves). But this interpretation ignores the fact that the large negative wave (presumably with its microwaves) is in reality induced in the superficial neurons by nervous transmission from the deep neurons, already firing during the first surface-positive (depth-negative) wave, as was already explained under Results. The above considerations lead us to the inference that the microwaves yield the large waves of the spike by the

process of summation; the inference is supported by the fact that the microwaves (as recorded by the monopolar method) are monophasic and hence might be summated; the monophasic characters of the microwaves is shown in the surface microelectrode record in Fig. 8 *A*. Our inference applies to summation of microwaves in the production of the first positive (depth-negative) wave and the large negative wave. Further support for our inference is afforded by Fig. 7 *C*; here the microwaves augment before a spike and decrease after it; during the interspike period the microwaves are small or imperceptible. Hence periodic augmentation of the microwaves would induce a train of spikes. It may be mentioned in this connection that Adrian and Matthews (3) regard the 'slow waves' of the electrocorticogram (without strychnine) as being caused by summation of the 'brief waves'. Our own conclusion is thus in harmony with the opinion of these writers.

Action of Strychnine and Cortical Histology

We shall now endeavor to relate the action of strychnine to the histological structure of the cortex; we shall first consider the strychnine spike. Our interpretation is based on Lorente de Nó's Fig. 74 in Fulton's *Physiology of the Nervous System* (21); Lorente de Nó has stated to one of us (F.R.M.) that this figure represents the basic pattern of the mammalian cortex. Now it is generally held that synchronization of neurons is a characteristic property of strychnine (Dusser de Barenne, Bremer) and this synchronization directs our attention to the cells with short axons (Golgi type II), which are present in every cortical layer; the axon of each cell forms baskets round a number of neurons; hence the discharge of a Golgi cell would induce discharges, practically synchronous, of a number of controlled neurons: here then is a basis for neuronal synchronization.

According to Dusser de Barenne and McCulloch, the strychnine spike originates in the deep pyramids of the Vth layer. Let us consider a Golgi cell in this layer: strychnine would stimulate its numerous afferent synapses and this would result in repetitive firing of its dendrites, soma, and axon; hence there would be synchronized, repetitive firing of the axon-controlled group of deep pyramids; through summation the effects, particularly in the somata, would appear as the first surface-positive (depth-negative) wave; the synchronous, repetitive firing of the axons of the deep pyramids would induce firing of the motor units, as shown in our experiments. Further, the axon of a deep pyramid gives off a recurrent collateral, which extends upwards into the superficial layers, and may be supposed to end in afferent synapses of Golgi cells; repetitive firing of the collateral would induce similar firing of the Golgi cells in these layers, including their axons, which would evoke repetitive, synchronous firing of the controlled (superficial) neurons; the result would be the large negative wave. It is obvious that the electrical potentials from these neurons would be much greater than those of the deep pyramids, because of space amplification through the interpolation of a multitude of Golgi cells in the superficial layers; also because of the greater neuronal population of

these layers; hence there is the remarkable amplitude of the surface-negative wave, which is the result of summation of microwaves (repetitive firing). Decrease of firing of the superficial neurons would then result in the descent of the surface-negative wave, ending in the final, smooth surface-positive wave, with relative neuronal quiescence.

Under Results we have expressed the opinion that the ascent of the surface-negative wave is excitomotor, in addition to the first surface-positive wave; the histological basis is furnished by Lorente de Nó's Fig. 74, referred to above; it will be recalled that the surface-negative wave and its microwaves are generated in the superficial neurons; in the figure pyramid 2, in a superficial layer, sends, from its descending axon, a collateral, which forms synapse S_4 on a dendrite of deep pyramid 8, which is thereby excited and emits, by its axon, efferent impulses to muscle. Thus a pathway would be afforded via the deep pyramids for excitomotor impulses arising in the superficial neurons.

As already stated, the progress of excitation and conduction under strychnine begins in the deep pyramids and passes to the superficial neurons. The somata would be responding repetitively and would contribute mainly to the production of the microwaves. Obviously axons and dendrites would be conducting repetitively, in accordance with Adrian's principles; corticifugal and corticipetal conduction would clearly also be repetitive; nerve fiber conduction would contribute somewhat to the production of microwaves. Thus the cortex under strychnine would be the seat of a most complex pattern of potential pulsations. McCulloch (27) draws the illuminating inference that normal conduction through the cortex would follow essentially the course of that under strychnine, though at a slower tempo. We would extend McCulloch's inference by stating that the basis of this intricate progress is repetitive, successive neuronal activity. This view receives support from the presence of cortical microwaves apart from the action of strychnine (Fig. 10). Thus it appears that there is a pre-existing functional cortical pathway, which strychnine employs in an intensive manner. It is obvious that numerous other possible pathways must exist besides that revealed by strychnine.

The triphasic strychnine spike, viewed as an entity, has a number of significant characteristics. In the first place, it represents a convulsive activity of the cortex, as revealed by its excitation of muscles; it has already been compared to the convulsive activity of the strychninized spinal cord. The marked regularity of successive spikes in a train recalls the appearance of certain rhythmical reflexes, for instance the series of reflex deglutitions, as induced by prolonged afferent stimulation of the superior laryngeal nerve (Zwaardemaker (37)). Now, swallowing involves a highly co-ordinated chain reaction, which can only be evoked by stimulation on the afferent side of the reflex arc; further, it shows a definite refractory phase (37).

The above considerations suggest that the elaborate sequence of intracortical conduction, underlying the strychnine spike, may also be interpreted as a co-ordinated chain reaction. The strychnine would act on the afferent side, namely on the afferent synapses of the Golgi cells; these would serve as

a pacemaker inducing the reaction, in the manner described under Histology above. The regularity of the rhythm in a train of spikes suggests the presence in the mechanism of a refractory phase, broadly comparable in principle to that in the deglutition reflex. Further, the regularity in amplitude of spikes in a series suggests that the reaction conforms to the all-or-none law: and this implies that the degree of involvement of the deep and superficial cortical layers tends to remain constant and must be attained by delicate co-ordination, as for instance, suitable adaptations of synaptic permeability.

Mode of Action of Eserine and ACh

As indicated above, there is a general similarity between the ACh and the strychnine cortical spikes: each is triphasic and each is probably built up by summation of microwaves; further, each spike shows phase reversal at the level of the deep pyramids. In earlier papers (30, 31, 32) reasons were given for believing that both eserine and ACh stimulate various synapses. In our present view ACh would stimulate the eserinized afferent synapses of Golgi cells in the Vth layer and thus induce the progressive conduction like that from strychnine. The action at the synapses by ACh may be a depolarization or a combination with protoplasmic receptors. However, as pointed out above, the basic chemical changes at the synapses would be different for strychnine and ACh, since the effects of ACh are suppressed by atropine, whereas those of strychnine are not thus suppressed. Despite this fundamental difference, it appears that strychnine and ACh are capable of triggering the same fundamental cortical mechanism of progressive excitation and conduction.

Human Epilepsy

Gibbs, Davis, and Lennox (22) recorded, in human epilepsy, cortical waves of high potential, which obviously evoke the muscular convulsions. These cortical waves, which we shall refer to as 'epileptic spikes', appear to be analogous to the spikes induced by strychnine and ACh: thus, allowing for differences in modes of electrode derivation, there is similarity in conformation, particularly as regards the large negative component. Also the epileptic spikes evoke convulsions, which are analogous to the excitation of motor units in our experiments.

We have expressed the opinion that the larger waves of the strychnine spike are built up by summation of microwaves, the latter representing fast, repetitive neuronal firing; since microwaves were observed during the ACh spike we may draw a similar conclusion as to the build-up of this from summation of microwaves. Thus we are led to speculate as to the likelihood that the epileptic spike may also be built up by summation of microwaves, representing fast, repetitive neuronal firing. A suggestion of this kind was made by Miller (34).

We come now to the question whether an excess of autogenous ACh may be an important factor in the etiology of epilepsy. A view of this kind was

expressed by Brenner and Merritt (10) and by Forster (20). Now ACh is a normal constituent of gray matter and it is often held to be concerned in synaptic transmission; in a concentration of 10^{-6} it evokes cortical spikes (reported in Introduction) and it is believed to stimulate various synapses. We may suppose that this substance, acting at synapses in abnormally high concentrations, would evoke fast microwaves (repetitive neuronal firing) and, by summation of these, the large epileptic spikes, with resulting convulsions. The convulsions would thus be induced fundamentally by the fast, repetitive neuronal firing.

From the above discussion we are led to the following conclusions. A drug which would lengthen the refractory period of the neuron would tend to reduce repetitive firing and thus should be of service in the treatment of epilepsy. In order to lessen the amount of autogenous ACh, cholinesterase substances would be indicated. Finally, since the ACh convulsive spikes are suppressed by intravenous atropine the probable value of drugs of the latter class is envisaged. It is of interest to recall that Belladonna has, at times, been employed in the treatment of epilepsy.

Acknowledgments

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The original drawings and one set of clear copies (e.g. small photographs) are to be submitted.

(iii) ***Photographs***

Prints should be made on glossy paper, with strong contrasts. They should be trimmed so that essential features only are shown and mounted carefully, with rubber cement, on white cardboard with no space or only a **very** small space (less than 1 mm.) between them. In mounting, full use of the space available should be made (to reduce the number of cuts required) and the ratio of height to width should correspond to that of a journal page (4¾ × 7½ in.); however, allowance must be made for the captions. Photographs or groups of photographs should not be more than 2 or 3 times the size of the desired reproduction.

Photographs are to be submitted in duplicate; if they are to be reproduced in groups one set should be mounted, the duplicate set unmounted.

Reprints

A total of 50 reprints of each paper, without covers, are supplied free. Additional reprints, with or without covers, may be purchased.

Charges for reprints are based on the number of printed pages, which may be calculated approximately by multiplying by 0.6 the number of manuscript pages (double-spaced typewritten sheets, 8½ × 11 in.) and including the space occupied by illustrations. An additional charge is made for illustrations that appear as coated inserts. The cost per page is given on the reprint requisition which accompanies the galley.

Any reprints required in addition to those requested on the author's reprint requisition form must be ordered officially as soon as the paper has been accepted for publication.

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